In compliance with the
Canadian Privacy Legislation
some supporting forms
may have been removed from
this dissertation.

While these forms may be included
in the document page count,
their removal does not represent
any loss of content from the dissertation.

UNIVERSITY OF ALBERTA

PRODUCTION, CHARACTERISATION AND EVALUATION OF *COPRINUS* PEROXIDASE FOR PHENOLIC WASTEWATER TREATMENT

BY

KEISUKE IKEHATA ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

ENVIRONMENTAL SCIENCE

DEPARTMENT OF CIVIL AND ENVIRONMENTAL ENGINEERING

EDMONTON, ALBERTA
FALL, 2003

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisisitons et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-612-87995-X
Our file Notre référence
ISBN: 0-612-87995-X

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou aturement reproduits sans son autorisation.

Canada

University of Alberta

Library Release Form

Name of Author: *Keisuke Ikehata*

Title of Thesis: *Production, characterisation and evaluation of* Coprinus *peroxidase for phenolic wastewater treatment*

Degree: *Doctor of Philosophy*

Year this Degree Granted: *2003*

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Sept. 25th, 2003

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled PRODUCTION, CHARACTERISATION AND EVALUATION OF *COPRINUS* PEROXIDASE FOR PHENOLIC WASTEWATER TREATMENT submitted by KEISUKE IKEHATA in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ENVIRONMENTAL SCIENCE.

Dr. David C. Ŝego

Date September 24 2003

Dedicated to my parents and my wife

Akihiro Ikehata

Terumi Ikehata

Keiko Ikehata

ABSTRACT

In this study, non-ligninolytic extracellular fungal peroxidases from *Coprinus* species were investigated as cost-effective alternatives to plant peroxidase for their application to phenolic wastewater treatment.

Based on a series of screening experiments, *C. cinereus* UAMH 4103 and *Coprinus* sp. UAMH 10067 and the peroxidases produced by these fungi were selected for further investigation. Batch productions of extracellular peroxidase by these fungi were successfully optimised through a series of growth experiments.

The extracellular peroxidases produced by these two *Coprinus* species were purified and characterised. Purified *Coprinus* peroxidases have a molecular weight of around 36 kDa based on MALDI-TOF mass spectrometry. The amino acid composition analyses of two *Coprinus* peroxidase revealed that they had very similar amino acid compositions, and that they were also similar to the previously studied *C. cinereus* peroxidase from different sources and also to *Arthromyces ramosus* peroxidase.

Although the catalytic properties of two *Coprinus* peroxidases were also very similar to each other, the thermal and pH stabilities of these enzymes were substantially different. The peroxidase from *Coprinus* sp. UAMH 10067 was more stable than that

from *C. cinereus* UAMH 4103 at an elevated temperature and under weakly acidic and weakly basic conditions. Both enzymes performed equally well in aqueous phenol removal around neutral pH; however, the peroxidase from *Coprinus* sp. UAMH 10067 worked better at pH 9 than did that from *C. cinereus* UAMH 4103.

When crude *Coprinus* peroxidases were used, the enzyme requirements to achieve 95% removal of 1.1 mM phenol from buffered solutions were 15 to 18 times smaller than those when purified ones were used. The crude *Coprinus* peroxidases showed similar phenol removal efficiencies to those of previously studied plant and fungal peroxidases. Treatment of real wastewater from an oil refinery with a crude *Coprinus* peroxidase was also successfully demonstrated in this study. These results indicate that crude *Coprinus* peroxidases, particularly that from *Coprinus* sp. UAMH 10067, have a potential for their large-scale production and application to phenolic wastewater treatment.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisors, Dr. Ian D. Buchanan and Dr. Daniel W. Smith for giving me the opportunity to do this work and for their encouragement and guidance throughout the course of this research. Especially, I would like to thank my advisors for offering me the opportunities for attending a number of international conferences, at which I could gain invaluable experience. I would also like to thank Dr. Michael A. Pickard for allowing me to work in his laboratory at the Department of Biological Sciences and for his help in this study. The help from Dr. Yuxin Wang in Dr. Pickard's laboratory is also gratefully acknowledged.

I would like to thank Mr. Werner Groeschel, Environmental Advisor, Imperial Oil, Strathcona Refinery for providing wastewater samples and technical information for this study.

My gratitude is expressed to my fellow graduate students, Mrs. Giyeon Yun and Mr. Xiaojian Mao, and laboratory technicians, Mrs. Debra Long, Mr. Gary Solonynko and Mr. Nick Chernuka, for their technical assistance in the laboratory. I would also like to thank Professor Lynne Sigler and Mrs. Arlene Flis of the University of Alberta Microfungus Collection and Herbarium and Dr. Akira Suzuki of Chiba University, Japan for their help in mycological experiments. I would also like to thank Dr. Suzuki for his critical reading on one of the chapters in this thesis and for introducing me the world of fungi. I would like to express my special gratitude to Mrs. Atsumi Hasimoto, Mr. Junichi Hashimoto, Dr. Yasuyuki Hiratsuka, Mr. Akira Nakamura, Mrs. Tomoko Suzuki, Dr. Akihiko Tsuneda and Mrs. Ichiko Tsuneda for their continuous friendships and encouragement, which made my life in Edmonton much more enjoyable and fruitful. The friendships, help and encouragement from many fellow graduate students in the program are also gratefully acknowledged.

I would like to thank my wife, Keiko Ikehata, for joining me from Japan, as well as for her tremendous help, encouragement and support over the course of this study. Without her deep understanding and indefatigable support, it was impossible for me to complete my study. Finally, I would like to thank my parents, Akihiro Ikehata and Terumi Ikehata, and my sister, Miyuki Ikehata, for their tremendous support and encouragement.

TABLE OF CONTENTS

Chapter 1. Introduction 1

1.1. Background 1

1.2. Objectives 4

References 6

Chapter 2. Literature Review of the Enzymatic Treatment of Phenols and Other Aromatic Compounds 12

2.1. Enzymatic treatment of phenols and other aromatic compounds 12

2.2. Peroxidases 12

2.2.1. Plant peroxidases 14

2.2.1.1. Horseradish peroxidase (HRP) 15

2.2.1.2. Soybean peroxidase (SBP) 18

2.2.1.3. Other plant peroxidases 19

2.2.2. Fungal peroxidases 20

2.2.2.1. Chloroperoxidase (CPO) 20

2.2.2.2. Lignin and manganese-dependent peroxidase (LiP and MnP) 21

2.2.2.3. Non-ligninolytic fungal peroxidase 22

2.2.3. Prokaryotic peroxidases 24

2.3. Polyphenol oxidases 25

2.3.1. Mushroom tyrosinase 25

2.3.2. Fungal laccase 27

References 30

Chapter 3. Recent Developments in the Production of Extracellular Fungal Peroxidases and Laccases for Waste Treatment 44

3.1. Introduction 44

3.2. Fungal peroxidases and fungal laccases 46

3.3. Lignin and manganese-dependent peroxidases (LiP and MnP) 48

3.3.1. Background 48

3.3.1.1. Catalytic activities of LiP and MnP 48

3.3.1.2. Application of LiP and MnP 50

3.3.2. Production of LiP and MnP 51

3.3.2.1. Earlier developments 51

3.3.2.2. Screening of fungi 52

3.3.2.3. Production optimisation 54

3.3.2.4. Phanerochaete *spp.* 58

3.3.2.5. Bjerkandera *spp.* 61

3.3.2.6. Pleurotus *spp.* 62

3.3.2.7. Other white-rot fungi 63

3.4. Non-ligninolytic fungal peroxidases 64

3.4.1. Background 64

3.4.1.1. Catalytic activity of non-ligninolytic fungal peroxidases 64

3.4.1.2. Application of non-ligninolytic fungal peroxidases 65

3.4.2. Production of non-ligninolytic fungal peroxidases 65

3.5. Fungal laccases 66

3.5.1. Background 66

3.5.1.1. Catalytic activities of laccase 67

3.5.1.2. Application of fungal laccases 69

3.5.2. Production of fungal laccases 70

3.5.2.1. Inducers 70

3.5.2.2. Use of waste products and solid-state fermentation 74

3.5.2.3. Selective breeding and strain selection 75

3.5.2.4. Optimisation of growth conditions 76

3.6. Concluding remarks 78

References 79

Chapter 4. Screening of *Coprinus* Species for the Production of Extracellular Peroxidase (Part I) 109

4.1. Introduction 109

4.2. Materials and methods 111

4.2.1. Materials 111

4.2.1.1. Fungi 111

4.2.1.2. Enzymes and chemicals 112

4.2.1.3. Equipment 113

4.2.2. Analytical methods 114

4.2.2.1. Peroxidase activity assay 114

4.2.2.2. Glucose assay 114

4.2.2.3. Hydrogen peroxide assay 115
4.2.2.4. Phenol assay 115
4.2.3. Experimental procedures 116
4.2.3.1. Peroxidase production experiments 116
4.2.3.2. Batch treatment experiments 116
4.3. Results and discussion 117
4.3.1. Production of extracellular fungal peroxidase 117
4.3.1.1. Screening of fungi 117
4.3.1.2. Time course of peroxidase production 120
4.3.2. Treatment of aqueous phenol with* Coprinus cinereus *peroxidase 123
4.3.2.1. Effects of initial peroxidase concentration and protective additives 123
4.3.2.2. Comparison of peroxidases 127
4.4. Conclusion 130
References 131

Chapter 5. Screening of *Coprinus* Species for the Production of Extracellular Peroxidase (Part II) 138
5.1. Introduction 138
5.2. Materials and methods 140
5.2.1. Fungi 140
5.2.2. Chemicals 140
5.2.3. Enzyme and chemical assays 141
5.2.4. Peroxidase production 141
5.3. Results and discussion 142
5.3.1. Screening of* Coprinus *species 142
5.3.2. Production of peroxidase by* Coprinus *sp. UAMH 10067 145
References 148

Chapter 6. Purification, Characterisation and Evaluation of Extracellular Peroxidase from Two *Coprinus* Species for Aqueous Phenol Treatment 152
6.1. Introduction 152
6.2. Materials and methods 155
6.2.1. Enzymes and chemicals 155

6.2.2. Fungi and peroxidase production **156**
6.2.3. Peroxidase activity assay **157**
6.2.4. Peroxidase purification **157**
6.2.5. Peroxidase characterisation **159**
6.2.6.* Coprinus *peroxidase activity as a function of pH and hydrogen peroxide concentration **159**
6.2.7. Stability of* Coprinus *peroxidase **159**
6.2.8. Batch treatment of aqueous phenol with* Coprinus *peroxidase **160**
6.2.9. Chemical assays **161**
6.3. Results **161**
6.3.1. Purification and characterisation of* Coprinus *peroxidase **161**
6.3.2.* Coprinus *peroxidase activity as a function of pH **165**
6.3.3.* Coprinus *peroxidase activity as a function of hydrogen peroxide concentration **165**
6.3.4. Stability of* Coprinus *peroxidase **170**
6.3.4.1. Effect of pH **170**
6.3.4.2. Effect of temperature **173**
6.3.5. Batch treatment of aqueous phenol with* Coprinus *peroxidase **173**
6.4. Discussion **177**
6.5. Conclusion **182**
References **184**

Chapter 7. Optimisation of Extracellular Fungal Peroxidase Production by Two *Coprinus* Species **190**
7.1. Introduction **190**
7.2. Materials and methods **192**
7.2.1. Fungi and general culture conditions **192**
7.2.2. Experimental designs for the optimisation of* Coprinus *peroxidase production **193**
7.2.2.1. Effects of carbon and nitrogen sources **193**
7.2.2.2. Screening of important factors **193**
7.2.2.3. Optimisation of glucose and peptone concentrations **195**
7.2.3. Enzyme and chemical assays **197**
7.2.4. Chemicals **198**

7.3. Results 198
- ***7.3.1. Effects of carbon and nitrogen sources on the peroxidase production by* Coprinus *species*** 198
- ***7.3.2. Screening of important factors for* Coprinus *peroxidase production*** 199
- ***7.3.3. Optimisation of glucose and peptone concentrations for* Coprinus *peroxidase production*** 203

7.4. Discussion 207

7.5. Conclusion 210

References 211

Chapter 8. Treatment of Oil Refinery Wastewater Using Crude *Coprinus cinereus* Peroxidase and Hydrogen Peroxide 214

8.1. Introduction 214

8.2. Materials and methods 217
- ***8.2.1. Materials*** 217
  - *8.2.1.1. Enzymes* 217
  - *8.2.1.2. Chemicals* 218
  - *8.2.1.3. Oil refinery wastewater* 218
- ***8.2.2. Equipment*** 219
- ***8.2.3. Analytical methods*** 219
  - *8.2.3.1. Peroxidase activity assay* 219
  - *8.2.3.2. Hydrogen peroxide assay* 220
  - *8.2.3.3. Total phenol assay* 220
  - *8.2.3.4. Chemical and biochemical oxygen demands and colour measurement* 221
- ***8.2.4. Experimental procedures*** 221
  - *8.2.4.1. Batch treatment experiments* 221
  - *8.2.4.2. Colour and product removal experiments* 222

8.3. Results and discussion 223
- ***8.3.1. Effect of pH*** 224
- ***8.3.2. Effect of hydrogen peroxide concentration*** 225
- ***8.3.3. Effects of peroxidase concentration, type and purity of peroxidase, and PEG addition*** 227
- ***8.3.4. Impact of enzymatic treatment on the COD and BOD_5 of the wastewater*** 229

8.3.5. Colour and product removal by coagulation 233
8.4. Conclusions 237
References 239

Chapter 9. Summary and Conclusions 244
9.1. Screening of *Coprinus* species for extracellular peroxidase production 244
9.2. Purification and characterisation of *Coprinus* peroxidase 246
9.3. Evaluation of *Coprinus* peroxidase for aqueous phenol treatment 248
9.4. Optimisation of *Coprinus* peroxidase production 250
9.5. Treatment of oil refinery wastewater using crude *Coprinus* peroxidase 251
9.6. Conclusions 253
9.7. Recommendations 256
References 258

Appendices 260
Appendix 1. Description of Fungi 261
Appendix 2. Production of Ligninolytic Enzymes by *Coprinus* Species 267
Appendix 3. Tabulated Data for Regression Analyses 270
A3.1. Psudo-steady state model for peroxidase activity 270
References 274
A3.2. Response surface analyses data 275
Appendix 4. MALDI-TOF Mass Spectra 277
Appendix 5. Amino Acid Analysis Data 279

LIST OF TABLES

Table 1.1. Examples of the enzymes studied for the removal of phenols and aromatic amines. 2

Table 2.1. Comparison of the enzymes evaluated for phenolic wastewater treatment. 30

Table 3.1. Some fungi recently studied for the production of extracellular peroxidase and laccase. 55

Table 3.2. Solid substrates and other waste products recently evaluated for the production of LiP, MnP and laccase. 59

Table 3.3. Examples of inducers recently evaluated for laccase production. 72

Table 4.1. List of fungi 112

Table 5.1. Peroxidase production by *Coprinus* species grown in YM broth for 10 days. 143

Table 6.1. Purification of peroxidase from *Coprinus* sp. UAMH 10067. 162

Table 6.2. Purification of peroxidase from *C. cinereus* UAMH 4103. 162

Table 6.3. Amino acid compositions of peroxidase from *Coprinus* sp. UAMH 10067, *C. cinereus* UAMH 4103, and published values for CIP and ARP. 164

Table 6.4. Estimated kinetic model parameters for the peroxidase activity of the peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 at pH 7.4 and 25°C. Standard errors are presented for the estimates of α, β and γ. 170

Table 6.5. Comparison of kinetic parameters for *Coprinus* peroxidase, soybean peroxidase and horseradish peroxidase at pH 7.4 and 25°C. 179

Table 6.6. Comparison of peroxidase requirement for the removal of 1 mM phenol at pH 7 and 25°C. 183

Table 7.1. Effect of carbon and nitrogen sources on the peroxidase production by *Coprinus* sp. UAMH 10067. Initial medium pH was adjusted at 6.2. 194

Table 7.2. 2^{7-3}_{IV} Fractional factorial design for the screening experiment. 195

Table 7.3. Design matrix and the response (maximum peroxidase activity in U mL^{-1}) of the central composite design for the optimisation of peroxidase production by *Coprinus* sp. UAMH 10067. 196

Table 7.4. Design matrix and the response (maximum peroxidase activity in U mL^{-1}) of the central composite design for the optimisation of peroxidase production by *C. cinereus* UAMH 4103. **197**

Table 7.5. Design matrix and the response (maximum peroxidase activity in U mL^{-1}) of the fractional factorial experiment. **200**

Table 7.6. Estimated main effects and two-factor interactions for the fractional factorial experiment. **201**

Table 7.7. Estimated model coefficients calculated from the results of the central composite design experiments. Standard errors of the estimated coefficients are also shown. **204**

Table 7.8. Calculated optimum concentrations of glucose and peptone for the production of peroxidase by two *Coprinus* species, and the responses derived from the models and the confirmation growth experiments. **205**

Table 8.1. Characteristics of oil refinery wastewater. **219**

Table A1.1. Description of fungi. **261**

Table A3.1. Apparent and relative peroxidase activity at various H_2O_2 concentrations (crude peroxidase from *Coprinus* sp. UAMH 10067). **272**

Table A3.2. Apparent and relative peroxidase activity at various H_2O_2 concentrations (purified peroxidase from *Coprinus* sp. UAMH 10067). **273**

Table A3.3. Apparent and relative peroxidase activity at various H_2O_2 concentrations (crude peroxidase from *C. cinereus* UAMH 4103). **273**

Table A3.4. Apparent and relative peroxidase activity at various H_2O_2 concentrations (purified peroxidase from *C. cinereus* UAMH 4103). **274**

Table A3.5. Complete data of the central composite design experiment for peroxidase production by *Coprinus* sp. UAMH 10067. **275**

Table A3.6. Complete data of the central composite design experiment for peroxidase production by *C. cinereus* UAMH 4103. **276**

Table A5.1. Amino acid analyses data for peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103. **279**

LIST OF FIGURES

Figure 2.1. Catalytic activity of a typical non-specific peroxidase. P-670 is a permanently inactivated form of enzyme called verodohaemoprotein. **13**

Figure 2.2. Schematic diagram of radical coupling and polymer formation following the peroxidase-catalysed oxidation of phenol. Two examples of dimer structures adapted from Yu *et al.* (1994) are shown. **16**

Figure 2.3. Catalytic cycles for the (I) hydroxylation of monophenols and (II) dehydrogenation of *o*-diphenols to *o*-quinones by tyrosinase. M = monophenol and D = diphenol bound forms (after Ikehata and Nicell, 2000). **26**

Figure 2.4. Simplified catalytic cycle of laccase for the four-electron reduction of O_2 to H_2O (after Solomon *et al.*, 1996). **28**

Figure 2.5. Typical diphenol oxidation catalysed by laccase (Thurston, 1994). **29**

Figure 4.1. Peroxidase activity in the liquid cultures of *Arthromyces ramosus* and *Coprinus* species after 5 and 11 days of growth at 25°C. **118**

Figure 4.2. Growth of *C. cinereus* and its production of peroxidase at 25°C: (a) UAMH 4103, (b) UAMH 7907 and (c) IFO 30116. **121**

Figure 4.3. Residual phenol as a function of initial CIP activity: $[\text{Phenol}]_0 =$ 1.1 mM, $[H_2O_2]_0 = 1.5$ mM, in 0.1 M sodium phosphate buffer at pH 7 at 25°C. **125**

Figure 4.4. Phenol transformed as a function of the H_2O_2 consumed with various amounts of CIP: $[\text{Phenol}]_0 = 1.1$ mM, $[H_2O_2]_0 = 1.5$ mM, in 0.1 M sodium phosphate buffer at pH 7 at 25°C. **125**

Figure 4.5. Colour formation as a function of initial CIP activity: $[\text{Phenol}]_0 =$ 1.1 mM, $[H_2O_2]_0 = 1.5$ mM, in 0.1 M sodium phosphate buffer at pH 7 at 25°C. Inset, the visible spectrum of reaction solutions: $[\text{CIP}]_0 = 1.4$ U mL^{-1}. **126**

Figure 4.6. Comparison of three enzymes on the phenol transformation: $[\text{Phenol}]_0 = 1.1$ mM, $[H_2O_2]_0 = 1.5$ mM, $[\text{peroxidase}]_0 = 0.4$ U mL^{-1}, in 0.1 M sodium phosphate buffer at pH 7 at 25°C. **128**

Figure 5.1. Production of peroxidase by *Coprinus* sp. UAMH 10067 in YM broth at 25°C. Mean of triplicates and standard deviation are shown. 145

Figure 5.2. Comparison of the stability of crude peroxidases from *Coprinus cinereus* UAMH 4103 and *Coprinus* sp. UAMH 10067 at (a) 25°C and (b) 50°C in 0.1 M phosphate buffer at pH 7.0. Initial peroxidase activity was 1.3 U mL^{-1}. Average values of duplicated test results are shown. 147

Figure 6.1. Catalytic cycle of peroxidase. P-670 is a permanently inactivated form of enzyme called verodohaemoprotein. 154

Figure 6.2. SDS-PAGE of purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 (lane 1: MW marker, lane 2: UAMH 10067 fraction #1, lane 3: UAMH 10067 fraction #2, lane 4: UAMH 4103, lane 5: ARP from Sigma). 163

Figure 6.3. Effect of pH on the activity of peroxidases from (a) *Coprinus* sp. UAMH 10067 and (b) *C. cinereus* UAMH 4103. Average values of duplicated assays of two peroxidase solutions with different enzyme concentrations (approximately 0.015 U mL^{-1} and 0.030 U mL^{-1}) are shown. 166

Figure 6.4. Effect of hydrogen peroxide concentration on the peroxidase activity of *Coprinus* peroxidase: (a) crude enzyme from *Coprinus* sp. UAMH 10067, (b) purified enzyme from *Coprinus* sp. UAMH 10067, (c) crude enzyme from *C. cinereus* UAMH 4103, and (d) purified enzyme from *C. cinereus* UAMH 4103. 167

Figure 6.5. pH stability of the peroxidases from *Coprinus* sp. UAMH 100671: (a) crude, (b) purified, and that from *C. cinereus* UAMH 4103: (c) crude, (d) purified at 25°C. Initial peroxidase activity was approximately 0.015 U mL^{-1}. Average values of two samples are shown. 171

Figure 6.6. Thermal stability of the crude and purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 at 50°C at pH 7. Initial peroxidase activity was approximately 0.015 U mL^{-1}. Average values of two samples are shown. 174

Figure 6.7. Aqueous phenol removal as a function of initial peroxidase activity using crude and purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 in the presence and absence of 200 mg L^{-1} PEG at 25°C at pH 7. ($[phenol]_0 = 1.1$ mM, $[H_2O_2]_0 = 1.5$ mM). 175

Figure 6.8. Effect of pH on the phenol removal using purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 at 25°C. ([phenol]$_0$ = 1.1 mM, [H_2O_2]$_0$ = 1.5 mM, [PEG]$_0$ = 200 mg L^{-1}). 176

Figure 7.1. Response surface of the central composite design experiment for the peroxidase production by *Coprinus* sp. UAMH 10067. 206

Figure 7.2. Response surface of the central composite design experiment for peroxidase production by *C. cinereus* UAMH 4103. 206

Figure 7.3. Growth of *Coprinus* sp. UAMH 10067 in the optimised medium and its peroxidase production. 208

Figure 7.4. Growth of *C. cinereus* UAMH 4103 in the optimised medium and its peroxidase production. 208

Figure 8.1. Residual total phenol in the oil refinery wastewater as a function of reaction pH after 2 hours of reaction and subsequent coagulation with 400 mg L^{-1} of alum at 18 ± 1°C ([Total Phenol]$_0$ = 6.4 mM, [H_2O_2]$_0$ = 10 mM, [CIP]$_0$ = 1.5 U mL^{-1}). 224

Figure 8.2. Effect of initial hydrogen peroxide concentration on residual total phenol, hydrogen peroxide and CIP activity in the oil refinery wastewater after 2 hours of reaction and subsequent coagulation with 400 mg L^{-1} of alum at 18 ± 1°C ([Total Phenol]$_0$ = 6.4 mM, [CIP]$_0$ = 4.3 U mL^{-1}). 226

Figure 8.3. Residual total phenol in the oil refinery wastewater, after 2 hours of reaction and subsequent coagulation with 400 mg L^{-1} of alum at 18 ± 1°C, as a function of initial peroxidase activity and the type of enzyme in the presence (closed symbols) and in the absence (open symbols) of 200 mg L^{-1} PEG ([Total Phenol]$_0$ = 6.4 mM, [H_2O_2]$_0$ = 10 mM). 228

Figure 8.4. COD and BOD_5 of the oil refinery wastewater treated with (a) crude CIP or (b) purified ARP at 18 ± 1°C before and after coagulation with 400 mg L^{-1} of alum (NBCOD: not-readily biodegradable COD (= Total COD – BOD_5), [Total Phenol]$_0$ = 6.4 mM, [H_2O_2]$_0$ = 10 mM). The "BC" and "AC" notation in the figure represents before and after coagulation, respectively. Error bars represent standard deviation of two treatments. 231

Figure 8.5. Visible spectra of the oil refinery wastewater treated with CIP at 18 ± 1°C before and after coagulation with 400 mg L^{-1} of alum, and after 24 hours without coagulation ([Total Phenol]$_0$ = 6.4 mM, $[H_2O_2]_0$ = 10 mM, $[CIP]_0$ = 3.5 U mL^{-1}). 234

Figure 8.6. Relationships between transformed total phenol and absorbance at 410 nm ($Abs_{410\ nm}$) before coagulation as well as COD removed after coagulation in the oil refinery wastewater treated with various amounts of CIP at 18 ± 1°C ([Total Phenol]$_0$ = 6.4 mM, $[H_2O_2]_0$ = 10 mM, $[CIP]_0$ = 0.5 – 2.5 U mL^{-1}, alum dose = 400 mg L^{-1}). 235

Figure 8.7. Removal of coloured products from the oil refinery wastewater treated with CIP at 18 ± 1°C as a function of alum dose for three initial total phenol concentrations (3.2, 6.4 and 9.6 mM). More than 98% of initial total phenol was transformed ($[H_2O_2]_0$ = 5 – 15 mM, $[CIP]_0$ = 2 – 8 U mL^{-1}). 237

Figure A2.1. Laccase activity in the liquid culture (YM broth) of *Coprinus* species grown at 25°C. 267

Figure A2.2. Laccase activity in the liquid culture (YM broth) of *Coprinus* species grown at 25°C for 10 days. 268

Figure A2.3. LiP activity in the liquid culture (YM broth) of *Coprinus* species grown at 25°C. 269

Figure A4.1. MALDI-TOF mass spectrum of peroxidase from *Coprinus* sp. UAMH 10067 (FPLC fraction #1). 277

Figure A4.2. MALDI-TOF mass spectrum of peroxidase from *Coprinus* sp. UAMH 10067 (FPLC fraction #2). 277

Figure A4.3. MALDI-TOF mass spectrum of peroxidase from *C. cinereus* UAMH 4103. 278

LIST OF SYMBOLS

Abbreviations

4-AAP: 4-Aminoantipyrine
ABTS: 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ARP: *Arthromyces ramosus* peroxidase
BOD_5: 5-Day biochemical oxygen demand
CIP: *Coprinus cinereus* peroxidase
COD: Chemical oxygen demand
CPO: Chloroperoxidase
HDCBS: 3,5-Dichloro-2-hydroxybenzenesulphonic acid sodium salt
HRP: Horseradish peroxidase
LCCD: Light catalytic cracked distillates
LiP: Lignin peroxidase
MALDI-TOF MS: Matrix assisted laser desorption ionisation time-of-flight mass spectrometry
MnP: Manganese-dependent peroxidase
NBCOD: Not-readily biodegradable COD (= total COD – BOD_5)
PEG: Poly(ethylene glycol)
SBP: Soybean peroxidase
SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Symbols

Chapter 6

C_a: Proportionality constant relating enzyme molar concentration to activity (mol U^{-1})
$[H_2O_2]_{maxR}$: Hydrogen peroxide concentration at which relative activity is maximum (mol L^{-1})
k_1: Reaction rate constant for hydrogen peroxide uptake by native peroxidase (mol^{-1} L s^{-1})
k_2: Reaction rate constant for the one-electron phenol oxidation by peroxidase Compound I (mol^{-1} L s^{-1})
k_3: Reaction rate constant for the one-electron phenol oxidation by peroxidase Compound II (mol^{-1} L s^{-1})

α: Model parameter (dimensionless)
β: Model parameter (mol L^{-1})
γ: Model parameter (mol^{-1} L)

Chapter 7

β_i: Regression coefficient for main effect
β_{ij}: Regression coefficient for interaction effect ($i \neq j$) or second-order effect ($i = j$)
η: Dependent variable of regression equation
b_i: Estimate of the regression coefficient for main effect
b_{ij}: Estimate of the regression coefficient for interaction effect ($i \neq j$) or second-order effect ($i = j$)
x_i: Level of experimental variable
y: Estimate of the dependent variable of regression equation

Chapter 1. Introduction

1.1. Background

Phenol, aromatic amines and their derivatives are often found in wastewaters from various industries such as coal conversion, petroleum refining, textile and dye, resins and plastics, wood preservation, and pharmaceutical and other organic chemical manufacturers. These compounds are often toxic to varieties of organisms including humans (Baker *et al.*, 1978; Swift, 1978), and some are suspected carcinogens or potential endocrine disrupting chemicals. Particularly, recent concerns regarding phenolic pollutants in aquatic environment are the phenolic endocrine disrupting chemicals, including alkylated phenols, such as 4-nonylphenol and 4-*tert*-octylphenol, and bisphenol A. These compounds are known to exert their effects at very low concentrations, as low as several micrograms per litre (Servos, 1999; Singleton and Khan, 2003).

Some of these aromatic compounds, especially polychlorinated and alkylated derivatives, are often resistant to the conventional biological treatment processes and tend to remain in biologically treated effluent and sludge (Maguire, 1999; La Guardia *et al.*, 2001). The presence of aromatic compounds, including phenols, in a drinking water source may lead to the formation of chlorinated compounds, which are generally more toxic, and also cause serious odour problems at very low concentrations, during the chlorination in a water treatment plant (WHO, 1996). In order to remove toxic phenols and aromatic amines from industrial wastewater, Klibanov *et al.* (1980) proposed an alternative treatment process using enzymes that catalyse oxidation of these compounds. In this enzymatic process, the aqueous phenols and aromatic amines are first oxidised to reactive free radicals or quinones by an enzyme, which is activated by an oxidant, such as hydrogen peroxide or molecular oxygen. The reactive free radicals and quinones are spontaneously polymerised to form water-insoluble polymers that can be removed from suspension by filtration or sedimentation. As shown in Table 1.1, enzymes including peroxidases, laccases and tyrosinases of plant and microbial origins have been found to be effective in this process (Karam and Nicell, 1997; Duran and Esposito, 2000).

Table 1.1. Examples of the enzymes studied for the removal of phenols and aromatic amines.

Enzyme	Source	Type	Reference
Horseradish peroxidase (HRP)	Horseradish roots	Plant peroxidase	Klibanov *et al.* (1980; 1983)
Soybean peroxidase (SBP)	Soybean hulls	Plant peroxidase	Caza *et al.* (1999), Wright and Nicell (1999)
Chloroperoxidase (CPO)	*Caldariomyces fumago*	Fungal peroxidase	Carmichael *et al.* (1985)
Lignin peroxidase (LiP)	White rot fungi	Fungal peroxidase	Aitken *et al.* (1989)
Manganese-dependent peroxidase (MnP)	White rot fungi	Fungal peroxidase	Aitken *et al.* (1994)
Coprinus cinereus peroxidase (CIP)	*C. cinereus* (*C. macrorhizus*)	Fungal peroxidase	Al-Kassim *et al.* (1994a; 1994b)
Arthromyces ramosus peroxidase (ARP)	*A. ramosus*	Fungal peroxidase	Ibrahim *et al.* (1997), Buchanan and Han (2000)
Laccase	White rot and other fungi	Fungal polyphenol oxidase	Bollag *et al.* (1988), Tatsumi *et al.* (1992)
Tyrosinase	Mushrooms (*Agarics bisporus*)	Fungal polyphenol oxidase	Atlow *et al.* (1984), Wada *et al.* (1993)

The enzymatic process has potential advantages over conventional biological processes and other technologies, such as chemical oxidation and activated carbon adsorption. For example, enzymes are highly selective, so that an enzymatic process can remove or detoxify specific pollutants, in this case phenols and anilines, over a wide range of concentrations in a complex matrix of pollutants (Karam and Nicell, 1997). They are less likely inhibited by the substances that are toxic to microorganisms. Unlike biological processes, there is no delay associated with the acclimation of biomass. The sludge production is also reduced because there is no biomass generation. Because the enzymes catalyse chemical reactions that would otherwise be too slow at ambient temperatures, the required retention time can be shorter and the reaction conditions can be milder and more environmentally benign than chemical treatment processes (Aitken, 1993).

Since the initial works of Klibanov and co-workers' (Klibanov *et al.*, 1980; Klibanov and Morris, 1981; Klibanov *et al.*, 1983) were reported, a great deal of research has been carried out to test the efficacy of enzymatic treatment of phenolic wastewater using various enzymes from different sources (Table 1.1) and to improve the process by modifying reaction conditions, evaluating various reactor configurations, using protective additives such as poly(ethylene glycol), talc or chitosan, and immobilising enzymes to solid supports to stabilise the enzyme's configuration and to allow it to be reused (Nicell, 2001). Although these studies have strongly indicated that enzymatic treatment has potential for large-scale application, further research is still required to improve its cost effectiveness. Especially, it is necessary to find economical enzyme sources and bulk enzyme production methods.

Of the plant and fungal enzymes listed in Table 1.1, horseradish peroxidase

(HRP) is one of the most comprehensively studied enzymes for phenolic wastewater treatment (Nicell, 2001). However, its production is limited because horseradish roots are cultivated mainly for the food industry and can be very costly for the bulk enzyme production required for wastewater treatment. Thus, other enzymes, including soybean peroxidase from soybean hulls, extracellular lignin peroxidases and laccases from wood degrading fungi, and other fungal peroxidases, have been investigated as alternatives to HRP (Aitken *et al.*, 1989; Tatsumi *et al.*, 1992; Al-Kassim *et al.*, 1994b; Wright and Nicell, 1999; Buchanan and Han, 2000). Although some of these enzymes are also commercially available, they are still too expensive for application to wastewater treatment (Duran and Esposito, 2000). This is mainly because the primary use of these enzymes, especially peroxidases, is aimed at clinical examination, such as immunoassay and glucose assay, and biochemical synthesis of chemical and pharmaceutical products that normally require highly purified enzymes. Because such high purity may not be needed for wastewater treatment (Cooper and Nicell, 1996; Roper *et al.*, 1996), the development of low-cost, low-purity enzyme production processes that are strictly designed for wastewater treatment is desired.

1.2. Objectives

The overall goal of this research is to identify a promising source of peroxidase for which a low cost production process may be developed to supply enzymes for the treatment of wastewaters containing phenolic and other aromatic compounds. Peroxidases have been chosen as primary enzymes because of their relatively broad specificity for the aromatic substrates (Karam and Nicell, 1997) and their relatively well-characterised properties (Dunford, 1999). More specifically, non-ligninolytic extracellular fungal peroxidases produced by such fungi as *Coprinus cinereus* and *Arthromyces ramosus* are targeted because of their potential for the bulk production, simple separation due to their extracellular nature, and the fact that this class of enzyme is relatively new to phenolic wastewater treatment.

Non-ligninolytic fungal peroxidases from *C. cinereus* and *A. ramosus* were first discovered in the late 1980's, and most of the characterisation studies on these enzymes had been carried out in the 1990's (Dunford, 1999). Several fungi have been reported to produce non-ligninolytic extracellular peroxidases, including a basidiomycete *C. cinereus* (also known as *C. macrorhizus*) (Morita *et al.*, 1988; DePillis and de Montellano, 1989), ascomycates *Cochliobolus* spp. and *Pellicularia* spp. (Ichikawa *et al.*, 1981) and a deuteromycete *A. ramosus* (Shinmen *et al.*, 1986). Only a limited number of reports have been published regarding non-ligninolytic peroxidase production using a few known strains of these fungi (Tsujimura *et al.*, 1994; Yao *et al.*, 1995; Sakurai *et al.*, 2002). It is known that *Coprinus* species are commonly found in many parts of the world (Orton and Watling, 1979), and there is a possibility to discover strains capable of producing non-ligninolytic fungal peroxidases more efficiently or to find strains producing enzymes with unique properties.

Therefore, in this study, a series of screening studies were performed to determine potential fungi for non-ligninolytic fungal peroxidase production from a number of *Coprinus* species collected from several culture collections, as well as from those recently isolated from the field (Chapters 4 and 5). The peroxidases obtained by liquid fermentation of selected fungi were separated from the biomass, purified and characterised for comparison with previously studied enzymes (Chapter 6). Some important properties of the crude and purified peroxidases, including catalytic activity and stability, for phenolic wastewater treatment were also studied (Chapter 6), and these enzymes were evaluated for aqueous phenol treatment and compared with previously studied plant and fungal peroxidases (Chapters 4 and 6). The productions of non-ligninolytic peroxidases by the selected fungi were optimised through a series of statistical experimental designs (Chapter 7). Finally, the use of peroxidases produced by the selected fungi for the treatment of real wastewater containing phenolic compounds was studied (Chapter 8). These chapters are preceded by literature reviews of enzymatic treatment of phenols and other aromatic compounds (Chapter 2) and of recent progress in the production of extracellular fungal enzymes, including the non-ligninolytic fungal peroxidase investigated in this study (Chapter 3).

References

Aitken, M. D. (1993). Waste treatment applications of enzymes: opportunities and obstacles. *The Chemical Engineering Journal* **52**: B49-B58.

Aitken, M. D., Massey, I. J., Chen, T. and Heck, P. E. (1994). Characterization of reaction products from the enzyme catalyzed oxidation of phenolic pollutants. *Water Research* **28**(9): 1879-1889.

Aitken, M. D., Venkatadri, R. and Irvine, R. L. (1989). Oxidation of phenolic pollutant by a lignin degrading enzyme from the white-rot fungus *Phanerochaete chrysosporium. Water Research* **23**(4): 443-450.

Al-Kassim, L., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1994a). Optimization of phenol removal by a fungal peroxidase from *Coprinus macrorhizus* using batch, continuous, and discontinuous semibatch reactors. *Enzyme and Microbial Technology* **16**: 120-124.

Al-Kassim, L., Taylor, K. E., Nicell, J. A., Bewtra, J. K. and Biswas, N. (1994b). Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxide from *Coprinus macrorhizus* in batch reactors. *Journal of Chemical Technology and Biotechnology* **61**: 179-182.

Atlow, S. C., Bonadonna-Aparo, L. and Klibanov, A. M. (1984). Dephenolization of industrial wastewaters catalyzed by polyphenol oxidase. *Biotechnology and Bioengineering* **26**: 599-603.

Baker, E. L., Landrigan, P. P., Bertozzi, P. E., Field, P. H., Basteyns, B. J. and Skinner, H. G. (1978). Phenol poisoning due to contaminated drinking water. *Archives of Environmental Health* **33**: 89-94.

Bollag, J.-M., Shuttleworth, K. L. and Anderson, D. H. (1988). Laccase-mediated detoxificaion of phenolic compounds. *Applied and Environmental Microbiology* **54**(12): 3086-3091.

Buchanan, I. D. and Han, Y. S. (2000). Assessment of the potential of *Arthromyces ramosus* peroxidase to remove phenol from industrial wastewaters. *Environmental Technology* **21**: 545-552.

Carmichael, R., Fedorak, P. M. and Pickard, M. A. (1985). Oxidation of phenols by chloroperoxidase. *Biotechnology Letters* **7**(4): 289-294.

Caza, N., Bewtra, J. K., Biswas, N. and Taylor, K. E. (1999). Removal of phenolic compounds from synthetic wastewater using soybean peroxidase. *Water Research* **33**(13): 3012-3018.

Cooper, V. A. and Nicell, J. A. (1996). Removal of phenols from a foundry wastewater using horseradish peroxidase. *Water Research* **30**(4): 954-964.

DePillis, G. D. and de Montellano, P. R. O. (1989). Substrate oxidation by the heme edge of fungal peroxidases. Reaction of *Coprinus macrorhizus* peroxidase with hydrazines and sodium azide. *Biochemistry* **28**(19): 7947-7952.

Dunford, H. B. (1999). *Heme Peroxidases.* New York, NY, John Wiley & Sons, Inc.

Duran, N. and Esposito, E. (2000). Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Applied Catalysis B: Environmental* **28**: 83-99.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997). Optimization of the removal of phenol from wastewater catalyzed by *Arthromyces ramosus* peroxidase. *CSCE/ACSE Environ. Eng. Conf.*, Edmonton, Alberta, Canada, Canadian Society for Civil Engineering, Montreal, Quebec. **2**: 1503-1510.

Ichikawa, K., Okazaki, K., Kimoto, K. and Watanabe, Y. (1981). Partial purification and characterization of peroxidase from *pellicularia filamentosa. Agricultural and Biological Chemistry* **45**(5): 1297-1299.

Karam, J. and Nicell, J. A. (1997). Potential application of enzymes in waste treatment. *Journal of Chemical Technology and Biotechnology* **69**: 141-153.

Klibanov, A. M., Alberti, B. N., Morris, E. D. and Felshin, L. M. (1980). Enzymatic removal of toxic phenols and anilines from waste waters. *Journal of Applied Biochemistry* **2**: 414-421.

Klibanov, A. M. and Morris, E. D. (1981). Horseradish peroxidase for the removal of carcinogenic aromatic amines from water. *Enzyme and Microbial Technology* **3**: 119-122.

Klibanov, A. M., Tu, T.-M. and Scott, K. P. (1983). Peroxidase-catalyzed removal of phenols from coal-conversion waste water. *Science* **221**: 259-261.

La Guardia, M. J., Hale, R. C., Harvey, E. and Mainor, T. M. (2001). Alkylphenol ethoxylate dagradation products in land-applied sewage sludge (biosolids). *Environmental Science and Technology* **35**(24): 4798-4804.

Maguire, R. J. (1999). Review of the persistence of nonylphenol and nonylphenol ethoxylates in aquatic environments. *Water Quality Research Journal of Canada* **34**(1): 37-78.

Morita, Y., Yamashita, H., Mikami, B., Iwamoto, H., Aibara, S., Terada, M. and Minami, J. (1988). Purification, crystallization, and characterization of peroxidase from *Coprinus cinereus*. *Journal of Biochemistry* **103**(4): 693-699.

Nicell, J. A. (2001). Environmental applications of enzymes. *Interdisciplinary Environmental Review* **3**(1): 14-41.

Orton, P. D. and Watling, R. (1979). Coprinaceae Part 1: Coprinus. in *British Fungus Flora Agarics and Boleti*. Henderson, D. M., Orton, P. D. and Watling, R., Editors. Royal Botanic Garden, Edinburgh, Her Majesty's Stationery Office: 7-125.

Roper, J. C., Dec, J. and Bollag, J.-M. (1996). Biodegradation and bioremediation: Using minced horseradish roots for the treatment of polluted waters. *Journal of Environmental Quality* **25**: 1242-1247.

Sakurai, A., Kawamoto, S., Abarca, J. F. and Sakakibara, M. (2002). Peroxidase production by *Coprinus cinereus* using rotating disk contactor. *Biochemical Engineering Journal* **10**: 47-53.

Servos, M. R. (1999). Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol ethoxylates. *Water Quality Research Journal of Canada* **34**(1): 123-177.

Shinmen, Y., Asami, S., Amachi, T., Shimizu, S. and Yamada, H. (1986). Crystallization and characterization of an extracellular fungal peroxidase. *Agricultural and Biological Chemistry* **50**(1): 247-249.

Singleton, D. W. and Khan, S. A. (2003). Xenoestrogen exposure and mechanisms of endocrine disruption. *Frontiers in Bioscience* **8**: S110-S118.

Swift, D. J. (1978). Some effects of exposing rainbow trout (*Salmo gairdneri* Richardson) to phenol solutions. *Journal of Fish Biology* **13**: 7-17.

Tatsumi, K., Wada, S., Ichikawa, H., Liu, S. Y. and Bollag, J.-M. (1992). Cross-coupling of a chloroaniline and phenolic acids catalyzed by a fungal enzyme. *Water Science and Technology* **26**(9-11): 2157-2160.

Tsujimura, H., Takaya, M., Katano, K., Matsumoto, N., Park, Y. S. and Okabe, M. (1994). Scaleup of peroxidase production by *Arthromyces ramosus* based on analysis of fluid velocity distribution. *Journal of Fermentation and Bioengineering* **77**(6): 650-654.

Wada, S., Ichikawa, H. and Tatsumi, K. (1993). Removal of phenols from wastewater by soluble and immobilized tyrosinase. *Biotechnology and Bioengineering* **42**(7): 854-858.

WHO (1996). Guidelines for Drinking-Water Quality, 2nd ed. Vol. 2 Health Criteria and Other Supporting Information. Geneva, World Health Organisation: 828-837.

Wright, H. and Nicell, J. A. (1999). Characterization of soybean peroxidase for the treatment of aqueous phenol. *Bioresource Technology* **70**(1): 69-79.

Yao, P.-X., Matsumoto, N., Tsujimura, H., Takaya, M. and Toda, K. (1995). Enhanced production of peroxidase in continuous culture of *Arthromyces ramosus* by selective bleeding of mycelium. *Journal of Fermentation and Bioengineering* **79**(4): 307-312.

Chapter 2. Literature Review of the Enzymatic Treatment of Phenols and Other Aromatic Compounds

2.1. Enzymatic treatment of phenols and other aromatic compounds

Enzymatic treatment of phenol and aromatic amines was first proposed using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) in early 1980's (Klibanov *et al.*, 1980). Variations of this process were also reported using peroxidases from other plant and fungal origins, as well as using polyphenol oxidases such as tyrosinase and laccase from various fungi (Table 1.1). Background of these enzymatic treatment processes, such as some aspects in phenol removal mechanisms, enzyme characteristics, and developments of the process, are summarised below for each class of enzymes, including plant peroxidases, fungal peroxidases, tyrosinase and fungal laccases.

2.2. Peroxidases

Peroxidases are a group of oxidoreductases that catalyse the reduction of peroxide such as H_2O_2 to water and the oxidation of a variety of organic and inorganic compounds. The term peroxidase represents a group of specific enzymes such as NAD peroxidase (EC 1.11.1.1), glutathione peroxidase (EC 1.11.1.9) and iodide peroxidase (EC 1.11.1.8), as well as a group of non-specific enzymes that are simply known as peroxidases (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7). With a few exceptions, the typical peroxidases are haemoproteins. Figure 2.1 illustrates the catalytic activity of a typical non-specific peroxidase such as HRP.

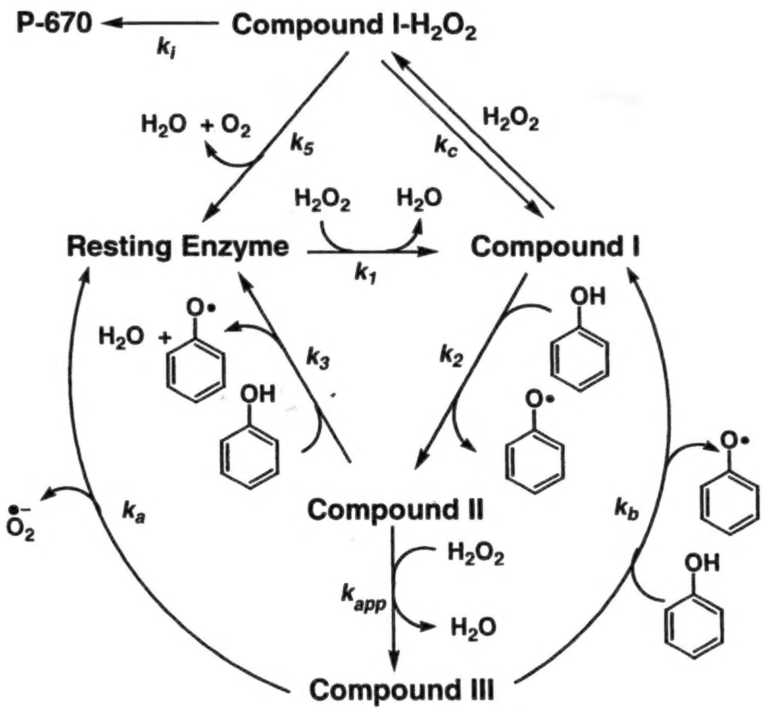

P-670
k_i
Compound I-H_2O_2
$H_2O + O_2$
k_5
k_c
H_2O_2
H_2O_2
H_2O
Resting Enzyme
k_1
Compound I
H_2O +
O•
OH
k_3
k_2
OH
O•
O•
$O_2^{•-}$
k_a
Compound II
k_b
H_2O_2
OH
k_{app}
H_2O
Compound III

Figure 2.1. Catalytic activity of a typical non-specific peroxidase. P-670 is a permanently inactivated form of enzyme called verodohaemoprotein.

The resting enzyme is first subject to a single two-electron oxidation by H_2O_2 and transformed to an active form called Compound I. Compound I oxidises one molecule of substrate (phenol molecules are shown here) by a one-electron oxidation to one molecule of reactive free radical, and transform itself to Compound II, another active form of peroxidase. Compound II is also capable of oxidising another molecule of substrate and return to the resting form. In the presence of excess H_2O_2, Compound II is oxidised to Compound III, which is slowly transformed to either resting enzyme or Compound I. Aromatic compounds including phenols, anilines, hydroquinones and hydroquinonoid amines, especially benzidine derivatives, are the known substrates of peroxidases (Josephy *et al.*, 1982; Putter and Becker, 1983). In the absence of such aromatic substrates, Compound I is oxidised by H_2O_2 to Compound I-H_2O_2, which finally leads to the permanent inactivation of enzyme (P-670 formation).

Among the haem-containing peroxidases, those from plants, fungi and bacteria share similar amino acid sequences and protein folds and constitute the plant peroxidase superfamily (Welinder, 1992). Although animals also produce peroxidases such as thyroid, lacto- and myeloperoxidases (Hosoya *et al.*, 1962), these enzymes are structurally unrelated to the peroxidases of plant, fungal and bacterial origins and constitute a separate superfamily. According to Welinder (1992), the plant peroxidase superfamily may be divided into three classes including prokaryotic peroxidases (class I), secretory fungal peroxidases (class II) and classical secretory plant peroxidases (class III). The peroxidases belonging to the plant peroxidase superfamily share similar catalytic activities, but are slightly different in terms of substrate specificities and subsequent reaction products. Brief summaries of enzyme characteristics and their applications to phenolic wastewater treatment are presented in the following subsections.

2.2.1. Plant peroxidases

Classical secretory plant peroxidases (class III) were isolated from varieties of plant species including horseradish (Welinder, 1976), turnip (Mazza and Welinder, 1980), tobacco (Lagrimini *et al.*, 1987), wheat (Rebmann *et al.*, 1991) and tomato (Roberts *et al.*, 1988). Each plant encodes 8 to 15 peroxidase families, some of which contain two or three highly similar genes. For example, in horseradish nine distinct genes or gene products have been sequenced and more than 40 isoenzymes have been detected (Dunford, 1991; Welinder, 1992). These enzymes have relatively narrower substrate specificity than do fungal lignin-degrading peroxidases. Many of the plant peroxidases are targeted to the endoplasmic reticulum and are sequestered in either the vacuole or the cell wall where they participate in the formation of rigid cross-links between cellulose, pectin, hydroxyproline-rich glycoproteins and lignin (Lagrimini *et al.*, 1987). Suberizing plant peroxidases are induced by wounding and implicated in the biosynthesis of the aromatic portion of suberin, a waxy waterproof substance deposited to seal the damaged tissue (Kolattukudy, 1984; Espelie *et al.*, 1986; Roberts *et al.*, 1988). Some peroxidases are also induced upon pathogenic attack of fungi and are implicated in the production of toxic compounds for defence (Score *et al.*, 1997). Several plant peroxidases have been evaluated for their applications to the enzymatic phenol removal proposed by Klibanov *et al.* (1980).

2.2.1.1. Horseradish peroxidase (HRP)

Klibanov *et al.* (1980) proposed a new enzymatic method to remove phenols and anilines from industrial wastewaters using HRP and H_2O_2. In this process, aqueous phenols and anilines are oxidised to free radicals in the presence of HRP and hydrogen peroxide (Figure 2.1). The free radicals are highly unstable in the aqueous solutions and spontaneously polymerise through radical coupling reactions to form precipitates of water-insoluble polymers (Figure 2.2). The precipitate formation occurs immediately after the addition of HRP and H_2O_2 into the solutions (Klibanov *et al.*, 1980), which implies that the oxidation of phenols and following spontaneous polymerisation are very quick. The coloured precipitates can be removed either by gravity separation or by filtration. The mechanism of the HRP-catalysed removal of phenols from aqueous solutions has successfully been modelled under various reaction conditions (Nicell, 1994; Buchanan and Nicell, 1998).

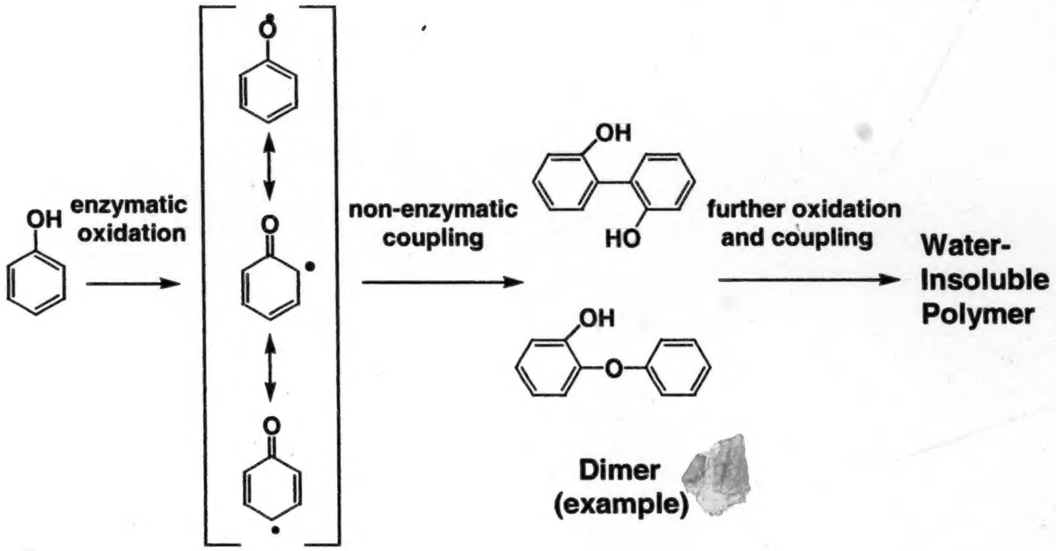

OH
enzymatic oxidation
non-enzymatic coupling
OH
HO
further oxidation and coupling
Water-Insoluble Polymer
OH
O
Dimer (example)

Figure 2.2. Schematic diagram of radical coupling and polymer formation following the peroxidase-catalysed oxidation of phenol. Two examples of dimer structures adapted from Yu *et al.* (1994) are shown.

A number of unsubstituted and substituted phenols and anilines have been successfully removed from aqueous solutions by HRP mediated treatment (Klibanov *et al.*, 1980). In addition, the efficient removal from artificial aqueous solutions of hydroxyl polyaromatic compounds such as 1-naphthol, biphenols such as *p*-phenylphenol, and aromatic amine carcinogens such as benzidines and naphthylamines has been demonstrated with high efficiency (Klibanov *et al.*, 1980; Klibanov and Morris, 1981). Although some compounds are not easily oxidised by HRP and hydrogen peroxide and require a higher dose of enzyme, the addition of an easily oxidised compound to the mixture has been shown to enhance the removal efficiency of hard to remove compounds through co-precipitation (Klibanov *et al.*, 1980; Roper *et al.*, 1995).

In addition to the artificial aqueous solutions, removal of phenolic compounds from real phenolic wastewaters from a variety of industries including coal conversion plants (Klibanov *et al.*, 1983), Kraft pulp mills (Paice and Jurasek, 1984; Davis and Burns, 1990; Wagner and Nicell, 2001b), semiconductor manufacturers (Nakamoto and Machida, 1992), foundries (Cooper and Nicell, 1996) and oil refineries (Wagner and Nicell, 2001a) have also been successfully treated with HRP and hydrogen peroxide in laboratory scale. Since real wastewaters tend to be a mixture of several aromatic compounds, it was suggested that better removal efficiency of these compounds might be expected from real wastewaters than from a pure solution of each compound (Klibanov *et al.*, 1980).

The reaction products from the HRP-catalysed oxidation of aqueous phenols have been characterised in terms of their structure (Yu *et al.*, 1994), relative hydrophobicity (Aitken *et al.*, 1994), and residual acute toxicity and mutagenicity (Aitken *et al.*, 1994; Massey *et al.*, 1994; Ghioureliotis and Nicell, 2000b). Yu *et al.* (1994) have identified five stable phenolic dimers and one trimer in aqueous solution during HRP-catalysed oxidation of phenol. These oligomers were subjected to further enzymatic oxidation and disappeared as the reaction proceeded. Aitken *et al.* (1994) have compared the characteristics of reaction products from enzymatic treatment of phenols with various peroxidase and phenol oxidases. According to their results of Microtox® assays, most of the reaction mixtures exerted higher toxicity than their parent compounds after the enzymatic treatment. It was also shown that the toxicity was mostly associated with soluble products rather than solid precipitates. Since reaction conditions such as pH and the enzymes used might affect the chemistry of reaction products, the authors suggested that controlling the reaction conditions of enzymatic treatment was necessary to achieve the detoxification of phenolic wastewaters.

As many researchers have pointed out, a major obstacle to the implementation of HRP-catalysed treatment of phenols to larger scales is the enzyme costs and the requirement of continuous enzyme dosing due to its inactivation. Several approaches have been reported to improve the cost effectiveness of the enzymatic treatment of phenolic wastewaters. For example, Nicell and co-workers have conducted the optimisation of reaction conditions such as pH, enzyme and H_2O_2 concentration, as well as the evaluation of various reactor configurations (Nicell *et al.*, 1992; Nicell *et al.*, 1993; Buchanan *et al.*, 1998). Nakamoto and Machida (1992) have shown that the use of non-ionic polymers such as gelatine and polyethylene glycol (PEG) greatly reduces HRP enzyme requirement by the factor of up to 200. Immobilisation of enzyme has also enhanced the removal efficiency and enabled the reuse of spent enzyme (Davis and Burns, 1990).

2.2.1.2. Soybean peroxidase (SBP)

Soybean peroxidase (SBP) is another plant peroxidase that has been studied recently for aqueous phenol treatment (Caza *et al.*, 1999; Wright and Nicell, 1999; Ghioureliotis and Nicell, 2000a; Ghioureliotis and Nicell, 2000b; Kinsley and Nicell, 2000). Similar to HRP, it was demonstrated that various phenolic substrates could be oxidised by SBP and H_2O_2 (Caza *et al.*, 1999; Wright and Nicell, 1999). Although SBP has significantly higher thermal stability, the catalytic reaction of SBP is slower than HRP and a larger molar quantity of enzyme is usually required to remove a given quantity of phenolic compounds (Wright and Nicell, 1999).

Although purified SBP is commercially available (Duran and Esposito, 2000), the production and use of crude preparations of the enzyme has drawn much attention because of the potential for cost reduction. Unlike HRP, which needs marketable horseradish roots as an enzyme source, SBP can be harvested from soybean hulls produced in large volumes as a waste product by food industries. Mantha *et al.* (2001) described an example of crude preparation of SBP from the hulls through extraction, centrifugation and filtration, as well as the use of crude SBP for the removal of nitrobenzene from synthetic wastewater in combination with Fe^0 reduction as a pre-treatment. Flock *et al.* (1999) evaluated the use of purified SBP as well as raw soybean hulls to remove aqueous phenol and 2-chlorophenol. These researchers noted that the use of raw soybean hulls is more effective than the use of purified SBP for phenol removal. It was assumed to be due to the absorption of polymerised products in the hulls as well as the slow leaching of SBP from the hulls.

2.2.1.3. Other plant peroxidases

Recently, the use of free and immobilised turnip peroxidase obtained from turnip roots was demonstrated to remove phenol from aqueous solution (Singh and Singh, 2002). Other plant peroxidases including root surface peroxidase from tomato and waterhyacinth (Adler *et al.*, 1994) were also found to be useful in removing phenolic compounds both *in vivo* and *in vitro* from water. Deposition of polymerised phenolic compounds onto the plant tissue was observed. Therefore, this approach is not a pure enzymatic process but rather is phytoremediation in which actual plants are used to remove pollutants from soil or from water. The authors suggested that the plant root might be used as a natural immobilised enzyme system for the remediation of phenolic compounds in the soil and water.

2.2.2. Fungal peroxidases

Peroxidases of fungal origins, studied for phenolic wastewater treatment, include chloroperoxidase (CPO; EC 1.11.1.10), lignin peroxidase (LiP, ligninase, diarylpropane peroxidase; EC 1.11.1.14), manganese-dependent peroxidase (MnP, manganese peroxidase; EC 1.11.1.13), *Coprinus cinereus* peroxidase (CIP; also known as *C. macrorhizus* peroxidase; EC 1.11.1.7) and *Arthromyces ramosus* peroxidase (ARP; EC 1.11.1.7). Among these fungal peroxidases, CPO is the only exception from class II fungal peroxidase proposed by Welinder (1992), because it has a distinct tertiary structure and catalytic activities from other plant and fungal peroxidases. Fungal peroxidases have been implicated in the control of growth (Brill, 1966), in the production of highly toxic compounds to protect against other fungal and bacterial attacks (Li, 1981), and in the induction of morphological changes (Score *et al.*, 1997). Some of the unique characteristics for each fungal peroxidase are presented below.

2.2.2.1. Chloroperoxidase (CPO)

Among these fungal peroxidases, CPO was discovered earliest from the culture of an imperfect fungus *Caldariomyces fumago* (Hager *et al.*, 1966; Morris and Hager, 1966). CPO catalyses at least three different reactions: halogenation of organic acceptor molecules such as β-keto acids, cyclic β-diketones and substituted phenols, the catalatic dismutation of hydrogen peroxide, and peroxidation (Hager *et al.*, 1966; Casella and Marchesini, 1994). It also catalyses other chemical reactions including epoxidation, sulfoxidation, oxidation of indoles and enantioselective propargylic hydroxylation (Littlechild, 1999). Carmichael *et al.* (1985) reported the oxidation of various phenolic compounds by CPO and subsequent removal of phenolic polymers from the solution as precipitates. Although the CPO-catalysed removal of phenols was comparable to that catalysed by HRP, the phenol treatment with the former enzyme leads to the formation of chlorinated products in the presence of chloride ions, which are often more toxic than parent compounds (Aitken *et al.*, 1994). This may be one disadvantage of the application of CPO to the treatment of industrial wastewaters that also contain chloride or other halide ions.

2.2.2.2. Lignin and manganese-dependent peroxidase (LiP and MnP)

Ligninolytic class II fungal peroxidases including LiP and MnP were discovered and isolated from extracellular culture medium of a white-rot basidiomycete *Phanerochaete chrysosporium* (Tien and Kirk, 1983; Kuwahara *et al.*, 1984; Tien and Kirk, 1984; Huynh and Crawford, 1985) and many other white rot fungi. Along with fungal laccase, LiP and MnP secreted by such white-rot fungi are responsible for the strong wood decomposing ability of these fungi. These enzymes are known to catalyse both polymerisation and degradation of lignin in the presence of hydrogen peroxide; however, the occurrence of the reactions is dependent on reaction conditions such as the existence of veratryl alcohol as a mediator and molecular weight of lignin molecules (Haemmerli *et al.*, 1986a; Kern and Kirk, 1987; Aitken and Irvine, 1989). More detailed review of catalytic properties of LiP and MnP is also presented in Chapter 3.

Because of the strong lignin degradation ability of white-rot fungi, a number of studies have been conducted to explore the application of the fungi themselves or the purified LiP and MnP from these fungi to the biochemical pulping of wood and to the decolourisation of bleach plant effluent (Eaton *et al.*, 1980; Sundman *et al.*, 1981; Trotter, 1990; Peralta-Zamora *et al.*, 1998). LiP and MnP from white-rot fungi have also been actively studied for the degradation of recalcitrant aromatic pollutants such as chlorinated phenols, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Haemmerli *et al.*, 1986b; Hammel *et al.*, 1986; Hammel and Tardone, 1988; Mileski *et al.*, 1988).

The application of these enzymes to phenolic wastewater treatment has been studied, because LiP and MnP are also capable of catalysing phenol oxidation (Aitken *et al.*, 1989; Aitken *et al.*, 1994). Aitken *et al.* (1994) reported that the molecular weight range of reaction products from LiP and MnP catalysed oxidation of phenolic compounds was wider than that from HRP catalysed oxidation, which was assumed to be due to the broader substrate specificity of former enzymes. This implies that controlling phenol removal using LiP or MnP may be more difficult. These enzymes also require addition of the co-substrates, veratryl alcohol for LiP and Mn^{+2} ion and certain types of buffers such as malonate for MnP, in order to allow HRP-like oxidation of phenolic compounds. This makes the use of these enzymes less practical for wastewater treatment.

2.2.2.3. Non-ligninolytic fungal peroxidase

Another type of class II fungal peroxidases, including ARP and CIP, that exhibit no ligninolytic activities were discovered in the mid- and late-1980s (Shinmen *et al.*, 1986; Morita *et al.*, 1988; DePillis and de Montellano, 1989). Comparison of molecular structures and catalytic activities of ARP and CIP indicates that they are identical, while the degrees of glycosylation of these proteins are slightly different (Kjalke *et al.*, 1992; Baunsgaard *et al.*, 1993; Sawai-Hatanaka *et al.*, 1995). Although these enzymes are more genetically related to other fungal peroxidases including LiP and MnP, they are very similar to classical plant peroxidases such as HRP rather than the ligninolytic peroxidases in substrate specificity, pH optimum (nearly neutral) and specific activity (Kjalke *et al.*, 1992; Baunsgaard *et al.*, 1993).

Non-ligninolytic fungal peroxidases including ARP and CIP are a relatively new class of enzymes in phenolic wastewater treatment. Al-Kassim *et al.* (1994b) reported that CMP (an identical enzyme of CIP) was capable of removing aqueous phenolic compounds in a similar manner to HRP. The authors have also conducted an optimisation study for the removal of aqueous phenol by CMP-catalysed oxidation in batch, semi-batch and continuous reactors in laboratory scale (Al-Kassim *et al.*, 1994a). The use of ARP in the treatment of aqueous phenols was also investigated in a similar manner to CMP (Al-Kassim *et al.*, 1993; Ibrahim *et al.*, 1997a; Ibrahim *et al.*, 1997b; Ibrahim *et al.*, 1997c). Buchanan and Han (2000) reported that the thermal stability of ARP was comparable to that of HRP but considerably less than that of SBP, while the phenol oxidative ability of ARP was as efficient as that of SBP but less efficient than that of HRP. A few groups of researchers also reported the application of CIP to the treatment of aqueous phenols (Kauffmann *et al.*, 1999; Masuda *et al.*, 2001a; Masuda *et al.*, 2001b; Masuda *et al.*, 2002). Other than aqueous phenol treatment, one report has recently been published regarding real wastewater (petroleum refinery wastewater) treatment using ARP (Ibrahim *et al.*, 2001). These studies have shown that non-ligninolytic fungal peroxidases can be used as cost effective alternatives to HRP for phenolic wastewater treatment, although more studies on enzyme production as well as on evaluation of these enzymes for real wastewater treatment will be needed.

2.2.3. Prokaryotic peroxidases

Although several class I prokaryotic peroxidases, including mitocondorial yeast cytochrome *c* peroxidase (EC 1.11.1.5), ascorbate peroxidase (EC 1.11.1.11) in plant chloroplasts and cytosol, in eukaryotic algae and in cyanobacteria and bacterial catalase-peroxidase (EC 1.11.1.6), were discovered and isolated a few decades ago, the environmental application of this class of peroxidase had not been documented until recently. Among these prokaryotic peroxidases, catalase-peroxidase is a dual-function peroxidase that catalyses both catalatic reactions in which hydrogen peroxide is decomposed to oxygen and water and peroxidative reactions in which classical peroxidase substrates such as phenol and aniline are oxidized (Claiborne and Fridovich, 1979). The use of this enzyme for phenolic waste treatment was proposed very recently.

Zamocky et al. (2001) investigated the expression and functioning of catalase and catalase-peroxidase during the biodegradation of phenol by a gram-negative rod-shaped bacterium, *Comamonas terrigena* N3H, which was isolated from contaminated soil. The authors found that the phenol degradation in the culture of *C. terrigena* was largely attributed to the reaction catalysed by catalase-peroxidase and proposed the use of this enzyme for the removal of phenolic compounds from the environment. The authors also suggested two potential advantages of the use of catalase-peroxidase for phenol removal over other plant and fungal peroxidases: (1) reduced formation of Compound III (see Figure 2.1), a notorious inactive form of peroxidase that forms in the presence of excessive hydrogen peroxide, due to its catalase activity, and (2) higher stability against mechanism based inactivation due to its oligomeric structure that protects the enzyme from the attack of reactive phenoxyl intermediates and from the inclusion by phenolic polymer products.

2.3. Polyphenol oxidases

Polyphenol oxidases are divided into two groups of enzymes, tyrosinase (monophenol, dihydroxyphenyalanine:dioxygen oxidoreductase, EC 1.14.18.1) and plant and fungal laccases (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2). Both of these enzymes contain multiple copper atoms at their active sites and utilise molecular oxygen to catalyse oxidation of varieties of phenols and other aromatic compounds to corresponding reactive quinones (Solomon *et al.*, 1996). Similar to the case of peroxidase-catalysed treatment of phenols, the reactive quinones couple to form large polymers that can be removed by coagulation or chemical adsorption.

2.3.1. Mushroom tyrosinase

Tyrosinase is widely distributed among the fungal, plant and animal kingdoms (Van Gelder *et al.*, 1997). The best-characterised tyrosinases are from fungi *Neurospora crassa* and *Agaricus bisporus* (mushroom). Tyrosinase contains a coupled binuclear copper active site. As shown in Figure 2.3, this enzyme catalyses two distinct oxidations of phenols. Molecular oxygen binds to the initial state of tyrosinase (E_{deoxy}) bringing it to an oxygenated state (E_{oxy}). Thereafter, the oxygenated tyrosinase catalyses the hydroxylation of monophenols in cycle I and the two-electron oxidation of *o*-diphenols to *o*-quinones in cycle II (Solomon *et al.*, 1996).

A variety of phenolic compounds are considered substrates of tyrosinase. In fungi and vertebrates, tyrosinase catalyses the initial step in formation of the pigment melanin from tyrosine (Rodriguez-Lopez *et al.*, 1992). In plants, tyrosinase oxidises a variety of phenolic compounds in the browning reaction observed when tissues are injured. One possible role of this browning reaction is the protection of the wound from pathogens and insects (Van Gelder *et al.*, 1997).

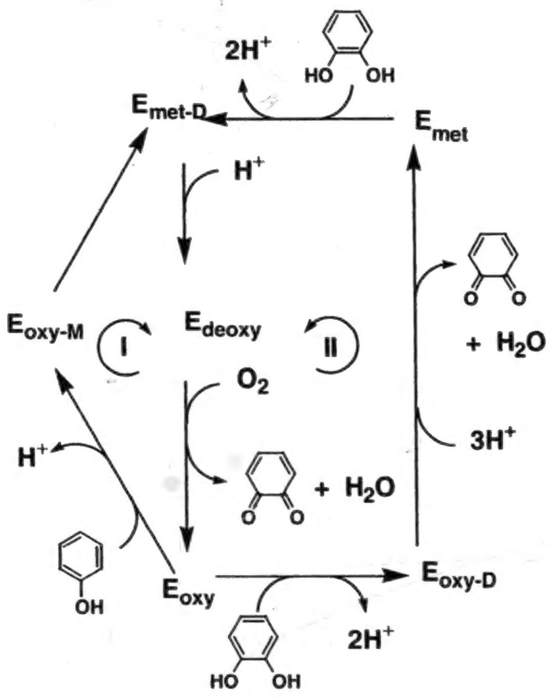


Figure 2.3. Catalytic cycles for the (I) hydroxylation of monophenols and (II) dehydrogenation of *o*-diphenols to *o*-quinones by tyrosinase. M = monophenol and D = diphenol bound forms (after Ikehata and Nicell, 2000).

Atlow *et al.* (1984) first reported the use of mushroom tyrosinase from *A. bisporus* for phenolic wastewater treatment. One major advantage of using tyrosinase is that tyrosinase utilises molecular oxygen as an oxidant for its catalytic activity, whereas peroxidase needs hydrogen peroxide for its activity. Tyrosinase catalyses the *ortho*-hydroxylation of phenol to catechols (*o*-diphenols) followed by the dehydrogenation of catechols to *o*-quinones. Since the *o*-quinones are not stable in aqueous solutions, they spontaneously oligomerised to produce pigment-like products.

A variety of phenols, aromatic amines, hydroxylated polyaromatic compounds were successfully oxidised by tyrosinase-catalysed reactions (Atlow *et al.*, 1984; Wada *et al.*, 1995). However, unlike peroxidase-catalysed reactions, the precipitation of reaction products was not observed but merely a coloured solution was obtained (Wada *et al.*, 1995; Ikehata and Nicell, 2000). These studies reported that addition of chitosan, partially deacetylated chitin, was needed to remove coloured products from the solution. Ikehata and Nicell (2000) conducted Microtox® acute toxicity assays on the reaction products from tyrosinase-catalysed oxidation of phenols. The toxicity of phenol solutions drastically reduced after the treatment with tyrosinase, whereas the toxicity increased after the treatment with peroxidases (Aitken *et al.*, 1994; Ghioureliotis and Nicell, 2000b).

2.3.2. Fungal laccase

Laccases have four copper atoms per molecule (Solomon *et al.*, 1996), and catalyse four electron reduction of O_2 to H_2O (Figure 2.4). Laccases can be divided further into two groups, plant and fungal laccases, although diphenol-oxidising enzymes that are thought to be laccases have also been identified in insects and bacteria (Thurston, 1994). Laccase is ubiquitous in fungi and has been detected and purified from many species such as *Trametes versicolor*, *Podospora anserine*, *Neurospora crassa*, and *Agaricus bisporus* (Bollag and Leonowicz, 1984; Thurston, 1994). Some of the fungi are known to produce multiple isoenzymes. Most of fungal laccases are extracellular, but some species may produce isoenzymes of both extra- and intracellular types (Solomon *et* *al.*, 1996).

A major physiological function of fungal laccases is lignin biodegradation. Along with ligninolytic peroxidases, including LiP and MnP, fungal laccase is believed to contribute to lignin degradation in nature. Other possible physiological functions of fungal laccases include morphogenesis, pigment formation, detoxification of toxic compounds, and plant pathogenesis (De Vries *et al.*, 1986; Thurston, 1994; Score *et al.*, 1997). More detailed properties of fungal laccases are also presented in Chapter 3.

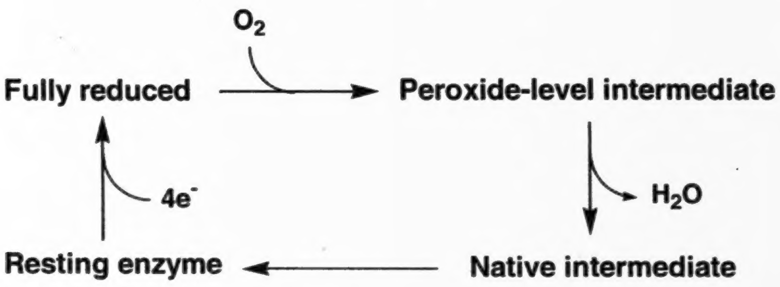


Figure 2.4. Simplified catalytic cycle of laccase for the four-electron reduction of O_2 to H_2O (after Solomon *et al.*, 1996).

Diphenols, such as hydroquinone and catechol, are the major substrates of laccase and are oxidised to free radicals by a one-electron reaction (Figure 2.5). The initial product is typically unstable and may either undergo a second oxidation to quinones or undergo non-enzymatic reactions such as disproportionation and/or polymerisation giving an amorphous insoluble melanin-like product (Thurston, 1994). Laccase acts like a battery, storing electrons from individual oxidation reactions in order to reduce molecular oxygen to water as shown in Figure 2.4. Laccase is also known to catalyse the oxidation of a range of substituted monophenols (except for tyrosine), polyphenols and other aromatic compounds resulting in demethylation, polymerisation and depolymerisation of the substrates (Coll *et al.*, 1993).

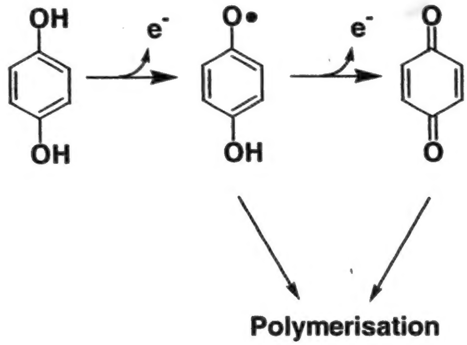


Figure 2.5. Typical diphenol oxidation catalysed by laccase (Thurston, 1994).

Fungal laccases obtained from various fungi have also been investigated for aqueous phenol treatment. As shown in Figures 2.4 and 2.5, laccase is first oxidised by molecular oxygen and subsequently catalyses the oxidation of diphenols to form reactive free radicals and quinones. Since laccase has a relatively broad specificity to its aromatic substrates, this enzyme is also known to catalyse the oxidation of a variety of monophenols and other aromatic compounds (Thurston, 1994). The products, both free radicals and quinones, are subjected to the non-enzymatic polymerisation.

Laccases from a soil fungus *Rhizoctonia praticola* and a white-rot fungus *Trametes versicolor* have also been investigated for the removal of a variety of phenols and anilines from aqueous solutions (Shuttleworth and Bollag, 1986; Simmons *et al.*, 1987; Bollag *et al.*, 1988; Milstein *et al.*, 1988; Simmons *et al.*, 1989; Roper *et al.*, 1995). Similar to LiP and MnP, because of their natural functions in lignin formation and/or delignification, fungal laccases have often been examined for the decolourisation of bleach plant effluent from pulp mills (Archibald *et al.*, 1990; Davis and Burns, 1992). Although the application of laccase to wastewater treatment is currently limited to pulp mill effluent, other types of phenolic wastewaters can be possibly treated with this enzyme.

To summarise this chapter, the advantages and disadvantages of the enzymes studied for phenolic wastewater treatment were compared and are presented in Table 2.1.

Table 2.1. Comparison of the enzymes evaluated for phenolic wastewater treatment.

Enzyme	Advantage	Disadvantage
HRP	Well-characterised catalytic activity	Expensive
SBP	High thermal stability, cheap (by-product of soybean industry)	Lower oxidative activity than HRP (high dose requirement)
CPO	Easy production	Formation of chlorinated products
LiP, MnP	Easy production, broad substrate specificity	Complex reaction mechanism (lignin degradation/polymerisation)
ARP, CIP	Easy production, high oxidative activity, simpler catalytic activity	More rapid inactivation than HRP
Mushroom tyrosinase	No peroxide requirement	Low oxidative activity, low thermal stability, coagulant requirement
Fungal laccase	Easy production, no peroxide requirement	Complex reaction mechanism (lignin degradation/polymerisation)

References

Adler, P. R., Arora, R., El Ghaouth, A., Glenn, D. M. and Solar, J. M. (1994). Bioremediation of phenolic compounds from water with plant root surface peroxidases. *Journal of Environmental Quality* **23**: 1113-1117.

Aitken, M. D. and Irvine, R. L. (1989). Stability testing of ligninase and Mn-peroxidase from *Phanerochaete chrysosporium. Biotechnology and Bioengineering* **34**: 1251-1260.

Aitken, M. D., Massey, I. J., Chen, T. and Heck, P. E. (1994). Characterization of reaction products from the enzyme catalyzed oxidation of phenolic pollutants. *Water Research* **28**(9): 1879-1889.

Aitken, M. D., Venkatadri, R. and Irvine, R. L. (1989). Oxidation of phenolic pollutant by a lignin degrading enzyme from the white-rot fungus *Phanerochaete chrysosporium. Water Research* **23**(4): 443-450.

Al-Kassim, L., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1993). Aromatic removal from water by *Arthromyces ramosus* peroxidase. in *Plant Peroxidases: Biochemistry and Physiology*. Welinder, K. G., Rasmussen, S. K., Penel, C. and Greppin, H., Editors, University of Geneva, Geneva: 197-200.

Al-Kassim, L., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1994a). Optimization of phenol removal by a fungal peroxidase from *Coprinus macrorhizus* using batch, continuous, and discontinuous semibatch reactors. *Enzyme and Microbial Technology* **16**: 120-124.

Al-Kassim, L., Taylor, K. E., Nicell, J. A., Bewtra, J. K. and Biswas, N. (1994b). Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxide from *Coprinus macrorhizus* in batch reactors. *Journal of Chemical Technology and Biotechnology* **61**: 179-182.

Archibald, F., Paice, M. G. and Jurasek, L. (1990). Decolorization of kraft bleachery effluent chromophores by *Coriolus (Trametes) versicolor. Enzyme and Microbial*

Technology **12**: 846-853.

Atlow, S. C., Bonadonna-Aparo, L. and Klibanov, A. M. (1984). Dephenolization of industrial wastewaters catalyzed by polyphenol oxidase. *Biotechnology and Bioengineering* **26**: 599-603.

Baunsgaard, L., Dalboge, H., Houen, G., Rasmussen, E. M. and Welinder, K. G. (1993). Amino acid sequence of *Coprinus macrorhizus* peroxidase and cDNA sequence encoding *Coprinus cinereus* peroxidase. A new family of fungal peroxidases. *European Journal of Biochemistry* **213**: 605-611.

Bollag, J.-M. and Leonowicz, A. (1984). Comparative studies of extracellular fungal laccases. *Applied and Environmental Microbiology* **48**(4): 849-854.

Bollag, J.-M., Shuttleworth, K. L. and Anderson, D. H. (1988). Laccase-mediated detoxificaion of phenolic compounds. *Applied and Environmental Microbiology* **54**(12): 3086-3091.

Brill, A. S. (1966). Peroxidases and catalase. in *Comprehensive Biochemistry*. Florkin, M. and Stotz, E. H., Editors. Amsterdam, Elsevier Publishing Company. **14**: 447-479.

Buchanan, I. D. and Han, Y. S. (2000). Assessment of the potential of *Arthromyces ramosus* peroxidase to remove phenol from industrial wastewaters. *Environmental Technology* **21**: 545-552.

Buchanan, I. D. and Nicell, J. A. (1998). Kinetics of peroxidase interactions in the presence of a protective additives. *Journal of Chemical Technology and Biotechnology* **72**: 23-32.

Buchanan, I. D., Nicell, J. A. and Wagner, M. (1998). Reactor models for horseradish peroxidase-catalyzed aromatic removal. *Journal of Environmental Engineering*

124(9): 794-802.

Carmichael, R., Fedorak, P. M. and Pickard, M. A. (1985). Oxidation of phenols by chloroperoxidase. *Biotechnology Letters* **7**(4): 289-294.

Casella, L. and Marchesini, A. (1994). The chloroperoxidase-catalyzed oxidation of phenols. Mechanism, selectivity and characterization of enzyme-substrate complexes. *Biochemistry* **33**(21): 6377-6386.

Caza, N., Bewtra, J. K., Biswas, N. and Taylor, K. E. (1999). Removal of phenolic compounds from synthetic wastewater using soybean peroxidase. *Water Research* **33**(13): 3012-3018.

Claiborne, A. and Fridovich, I. (1979). Purification of the *o*-dianisidine peroxidase from *Escherichia coli* B. Physicochemical characterization and analysis of its dual catalatic and peroxidatic activities. *The Journal of Biological Chemistry* **254**(10): 4245-4252.

Coll, P. M., Tabernero, C., Santamaria, R. and Perez, P. (1993). Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic basidiomycete PM1 (CECT 2971). *Applied and Environmental Microbiology* **59**(12): 4129-4135.

Cooper, V. A. and Nicell, J. A. (1996). Removal of phenols from a foundry wastewater using horseradish peroxidase. *Water Research* **30**(4): 954-964.

Davis, S. and Burns, R. G. (1990). Decolorization of phenlic effluents by soluble and immobilized phenol oxidases. *Applied Microbiology and Biotechnology* **32**: 721-726.

Davis, S. and Burns, R. G. (1992). Covalent immobilisation of laccase on activated carbon for phenolic effluent treatment. *Applied Microbiology and Biotechnology*

37: 474-479.

De Vries, O. M. H., Kooistra, W. H. C. F. and Wessels, J. G. H. (1986). Formation of an extracellular laccase by a *Schizophyllum commune* dikaryon. *Journal of General Microbiology* **132**: 2817-2826.

DePillis, G. D. and de Montellano, P. R. O. (1989). Substrate oxidation by the heme edge of fungal peroxidases. Reaction of *Coprinus macrorhizus* peroxidase with hydrazines and sodium azide. *Biochemistry* **28**(19): 7947-7952.

Dunford, H. B. (1991). Horseradish peroxidase: Structure and kinetic properties. in *Peroxidases in Chemistry and Biology*. Everse, J., Everse, K. E. and Grisham, M. B., Editors, CRC Press. **2**: 1-24.

Duran, N. and Esposito, E. (2000). Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Applied Catalysis B: Environmental* **28**: 83-99.

Eaton, D., Chang, H.-m. and Kirk, T. K. (1980). Fungal decolorization of kraft bleach plant effluents. *Tappi* **63**(10): 103-106.

Espelie, K. E., Franceschi, V. R. and Kolattukudy, P. E. (1986). Immunocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. *Plant Physiology* **81**: 487-492.

Flock, C., Bassi, A. and Gijzen, M. (1999). Removal of aqueous phenol and 2-chlorophenol with purified soybean peroxidase and raw soybean hulls. *Journal of Chemical Technology and Biotechnology* **74**(4): 303-309.

Ghioureliotis, M. and Nicell, J. A. (2000a). Assessment of soluble products of peroxidase-catalyzed polymerisation of aquous phenol. *Enzyme and Microbial*

Technology **25**: 185-183.

Ghioureliotis, M. and Nicell, J. A. (2000b). Toxicity of soluble products from the peroxidase-catalysed polymerization of substituted phenolic compounds. *Journal of Chemical Technology and Biotechnology* **75**: 98-106.

Haemmerli, S. D., Leisola, M. S. A. and Fiechter, A. (1986a). Polymerisation of lignins by ligninases from *Phanerochaete chrysosporium*. *FEMS Microbiology Letters* **35**: 33-36.

Haemmerli, S. D., Leisola, M. S. A., Sanglard, D. and Fiechter, A. (1986b). Oxidation of benzo(*a*)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. *The Journal of Biological Chemistry* **261**(15): 6900-6903.

Hager, L. P., Morris, D. R., Brown, F. S. and Eberwein, H. (1966). Chloroperoxidase. 2. Utilization of halogen anions. *The Journal of Biological Chemistry* **241**(8): 1769-1777.

Hammel, K. E., Kalyanaraman, B. and Kirk, T. K. (1986). Oxidation of polycyclic aromatic hydrocarbons and dibenzo[*p*]-dioxins by *Phanerochaete chrysosporium* ligniase. *The Journal of Biological Chemistry* **261**(36): 16948-16952.

Hammel, K. E. and Tardone, P. J. (1988). The oxidative 4-dechlorination of polychlorinated phenols is catalyzed by extracellular fungal lignin peroxidases. *Biochemistry* **27**(17): 6563-6568.

Hosoya, T., Kondo, Y. and Ui, N. (1962). Peroxidase activity in thyroid gland and partial purification of the enzyme. *The Journal of Biochemistry* **52**(3): 180-189.

Huynh, V.-B. and Crawford, R. L. (1985). Novel extracellular enzymes (ligninases) of *Phanerochaete chrysosporium*. *FEMS Microbiology Letters* **28**: 119-123.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997a).

Optimization of the removal of phenol from wastewater catalyzed by *Arthromyces ramosus* peroxidase. *CSCE/ACSE Environ. Eng. Conf.*, Edmonton, Alberta, Canada, Canadian Society for Civil Engineering, Montreal, Quebec. **2**: 1503-1510.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997b). Removal of phenol from industrial wastewater using *Arthromyces ramosus* peroxidase in a continuous flow system. *Proc. of the 52nd Indust. Waste Conf.*, Purdue University, West Lafayette, Indiana, Ann Arbor Press, Ann Arbor, Michigan: 271-277.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997c). Model development for the removal of phenol from water catalyzed by *Arthromyces ramosus* peroxidase. *International Association on Water Quality (IAWQ) Specialised Conference on Chemical Process Industries and Environmental Management*, Cape Town, South Africa.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (2001). Enzyme-catalyzed removal of phenol from refinery wastewater: Feasibility studies. *Water Environment Research* **73**(2): 165-172.

Ikehata, K. and Nicell, J. A. (2000). Color and toxicity removal following tyrosinase-catalyzed oxidation of phenols. *Biotechnology Progress* **16**(4): 533-540.

Josephy, P. D., Eling, T. and Mason, R. P. (1982). The horseradish peroxidase-catalyzed oxidation of 3,5,3',5'-tetramethylbenzidine. Free radical and charge-transfer complex intermediates. *The Journal of Biological Chemistry* **257**(7): 3669-3675.

Kauffmann, C., Petersen, B. R. and Bjerrum, M. J. (1999). Enzymatic removal of phenols from aqueous solutions by *Coprinus cinereus* peroxidase and hydrogen peroxide. *Journal of Biotechnology* **73**: 71-74.

Kern, H. W. and Kirk, T. K. (1987). Influence of molecular size and ligninase pretreatment on degradation of lignins by *Xanthomonas* sp. strain 99. *Applied and Environmental Microbiology* **53**(9): 2242-2246.

Kinsley, C. and Nicell, J. A. (2000). Treatment of aqueous phenol with soybean peroxidase in the presence of polyethylene glycol. *Bioresource Technology* **13**(2): 139-146.

Kjalke, M., Andersen, M. B., Schneider, P., Christensen, B., Schulein, M. and Welinder, K. G. (1992). Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. *Biochimica et Biophysica Acta* **1120**: 248-256.

Klibanov, A. M., Alberti, B. N., Morris, E. D. and Felshin, L. M. (1980). Enzymatic removal of toxic phenols and anilines from waste waters. *Journal of Applied Biochemistry* **2**: 414-421.

Klibanov, A. M. and Morris, E. D. (1981). Horseradish peroxidase for the removal of carcinogenic aromatic amines from water. *Enzyme and Microbial Technology* **3**: 119-122.

Klibanov, A. M., Tu, T.-M. and Scott, K. P. (1983). Peroxidase-catalyzed removal of phenols from coal-conversion waste water. *Science* **221**: 259-261.

Kolattukudy, P. E. (1984). Biochemistry and function of cutin and suberin. *Canadian Journal of Botany* **62**: 2918-2933.

Kuwahara, M., Glenn, J. K., Morgan, M. A. and Gold, M. H. (1984). Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. *FEBS Letters* **169**(2): 247-250.

Lagrimini, L. M., Burkhart, W., Moyer, M. and Rothstein, S. (1987). Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: Molecular analysis and tissue-specific expression. *Proceedings of the National Academy of Sciences of the United States of America* **84**(21): 7542-7546.

Li, C. Y. (1981). Phenoloxidase and peroxidase activities in zone lines of *Phellinus weirii*. *Mycologia* **73**: 811-821.

Littlechild, J. (1999). Haloperoxidases and their role in biotransformation reactions. *Current Opinion in Chemical Biology* **3**: 28-34.

Mantha, R., Biswas, N., Taylor, K. E. and Bewtra, J. K. (2001). Model for removal of nitrobenzene from synthetic waste water using 2-step Fe^0 reduction and peroxidase-catalyzed oxidation. *7th Speciality Conference on Environmental Engineering of the Canadian Society for Civil Engineering*, Victoria, BC, Canada, Canadian Society for Civil Engineering, Montreal, PQ: 6 pages (CDROM).

Massey, I. J., Aitken, M. D., Ball, L. M. and Heck, P. E. (1994). Mutagenicity screening of reaction products from the enzyme-catalyzed oxidation of phenolic pollutants. *Environmental Toxicology and Chemistry* **13**(11): 1743-1752.

Masuda, M., Sakurai, A. and Sakakibara, M. (2001a). Effect of reaction conditions on phenol removal by polymerization and precipitation using *Coprinus cinereus* peroxidase. *Enzyme and Microbial Technology* **28**: 295-300.

Masuda, M., Sakurai, A. and Sakakibara, M. (2001b). Effect of enzyme impurities on phenol removal by the method of polymerization and precipitation catalyzed by *Coprinus cinereus* peroxidase. *Applied Microbiology and Biotechnology* **57**:

494-499.

Masuda, M., Sakurai, A. and Sakakibara, M. (2002). Effect of temperature and pH on phenol removal using purified *Coprinus cinereus* peroxidase. *World Journal of Microbiology and Biotechnology* **18**: 739-743.

Mazza, G. and Welinder, K. G. (1980). Covalent structure of turnip peroxidase 7. *European Journal of Biochemistry* **108**: 481-489.

Mileski, G. J., Bumpus, J. A., Jurek, M. A. and Aust, S. D. (1988). Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **54**(12): 2885-2889.

Milstein, O., Haars, A., Majcherczyk, A., Trojanowski, J., Tautz, D., Zanker, H. and Huttermann, A. (1988). Removal of chlorophenols and chlorolignins from bleaching effluent by combined chemical and biological treatment. *Water Science and Technology* **20**(1): 161-170.

Morita, Y., Yamashita, H., Mikami, B., Iwamoto, H., Aibara, S., Terada, M. and Minami, J. (1988). Purification, crystallization, and characterization of peroxidase from *Coprinus cinereus*. *Journal of Biochemistry* **103**(4): 693-699.

Morris, D. R. and Hager, L. P. (1966). Chloroperoxidase. 1. Isolation and properties of the crystalline glycoprotein. *The Journal of Biological Chemistry* **241**(8): 1763-1768.

Nakamoto, S. and Machida, N. (1992). Phenol removal from aqueous solutions by peroxidase-catalyzed reaction using additives. *Water Research* **26**(1): 49-54.

Nicell, J. A. (1994). Kinetics of horseradish peroxidase-catalyzed polymerization and precipitation of aqueous 4-chlorophenol. *Journal of Chemical Technology and Biotechnology* **60**: 203-215.

Nicell, J. A., Bewtra, J. K., Biswas, N. and Taylor, K. E. (1993). Reactor development for peroxidase catalyzed polymerization and precipitation of phenols from wastewater. *Water Research* **27**(11): 1629-1639.

Nicell, J. A., Bewtra, J. K., Taylor, K. E., Biswas, N. and Pierre, C. S. (1992). Enzyme catalyzed polymerization and precipitation of aromatic compounds from wastewater. *Water Science and Technology* **25**(3): 157-164.

Paice, M. G. and Jurasek, L. (1984). Peroxidase-catalyzed color removal from bleach plant effluent. *Biotechnology and Bioengineering* **26**: 477-480.

Peralta-Zamora, P., Moraes, S. G. d., Esposito, E., Antunes, R., Reyes, J. and Duran, N. (1998). Decolorization of pulp mill effluents with immobilized lignin and manganese peroxidase from *Phanerochaete chrysosporium. Environmental Technology* **19**: 521-528.

Putter, J. and Becker, R. (1983). Peroxidases. in *Methods of Enzymatic Analysis*. Bergmeyer, H. U., Bergmeyer, J. and Grabl, M., Editors. Weinhem, Verlag Chemie. **3**: 286-293.

Rebmann, G., Hertig, G., Bull, J., Mauch, F. and Dudler, R. (1991). Cloning and sequencing of cDNAs encoding a pathogen-induced putative peroxidase of wheat (*Triticum aestivum* L.). *Plant Molecular Biology* **16**: 329-331.

Roberts, E., Kutchan, T. and Kolattukudy, P. E. (1988). Cloning and sequencing of cDNA for a highly anionic peroxidase from potato and the induction of its mRNA in suberizing potato tubers and tomato fruits. *Plant Molecular Biology* **11**: 15-26.

Rodriguez-Lopez, J. N., Tudela, J., Varon, R., Garcia-Carmona, F. and Garcia-Canovas, F. (1992). Analysis of a kinetic model for melanin biosynthesis pathway. *The Journal of Biological Chemistry* **267**(6): 3801-3810.

Roper, J. C., Sarkar, J. M., Dec, J. and Bollag, J.-M. (1995). Enhanced enzymatic removal of chlorophenols in the presence of co-substrates. *Water Research* **29**(12): 2720-2724.

Sawai-Hatanaka, H., Ashikari, T., Tanaka, Y., Asada, Y., Nakayama, T., Minakata, H., Kunishima, N., Fukuyama, K., Yamada, H., Shibano, Y. and Amachi, T. (1995). Cloning, sequencing, and heterologous expression of a gene coding for *Arthromyces ramosus* peroxidase. *Bioscience, Biotechnology, and Biochemistry* **59**(7): 1221-1228.

Score, A. J., Palfreyman, J. W. and White, N. A. (1997). Extracellular phenoloxidase and peroxidase enzyme production during interspecific fungal interactions. *International Biodeterioration & Biodegradation* **39**(2): 225-233.

Shinmen, Y., Asami, S., Amachi, T., Shimizu, S. and Yamada, H. (1986). Crystallization and characterization of an extracellular fungal peroxidase. *Agricultural and Biological Chemistry* **50**(1): 247-249.

Shuttleworth, K. L. and Bollag, J.-M. (1986). Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. *Enzyme and Microbial Technology* **8**: 171-177.

Simmons, K. E., Minard, R. D. and Bollag, J.-M. (1987). Oligomerization of 4-chloroaniline by oxidoreductases. *Environmental Science and Technology* **21**(10): 999-1003.

Simmons, K. E., Minard, R. D. and Bollag, J.-M. (1989). Oxidative co-oligomerization of guaiacol and 4-chloroaniline. *Environmental Science and Technology* **23**(1): 115-121.

Singh, N. and Singh, J. (2002). An enzymatic method for removal of phenol from industrial effluent. *Preparative Biochemistry and Biotechnology* **32**(2): 127-133.

Solomon, E. I., Sundaram, U. M. and Machonkin, T. E. (1996). Multicopper oxidases and oxygenases. *Chemical Reviews* **96**(7): 2563-2605.

Sundman, G., Kirk, T. K. and Chang, H.-m. (1981). Fungal decolorization of kraft bleach plant effluent. Fate of the chromophoric material. *Tappi* **64**(9): 145-148.

Thurston, C. F. (1994). The structure and function of fungal laccases. *Microbiology* **140**: 19-26.

Tien, M. and Kirk, T. K. (1983). Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. *Science* **221**: 661-663.

Tien, M. and Kirk, T. K. (1984). Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. *Proceedings of the National Academy of Sciences of the United States of America* **81**: 2280-2284.

Trotter, P. C. (1990). Biotechnology in the pulp and paper industry: A review. Part 1: tree improvement, pulping and bleaching, and dissolving pulp applications. *Tappi Journal* **73**(4): 198-204.

Van Gelder, C. W. G., Flurkey, W. H. and Wichers, H. J. (1997). Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry* **45**(7): 1309-1323.

Wada, S., Ichikawa, H. and Tatsumi, K. (1995). Removal of phenols and aromatic amines from wastewater by a combination treatment with tyrosinase and coagulant. *Biotechnology and Bioengineering* **45**(4): 304-309.

Wagner, M. and Nicell, J. A. (2001a). Peroxidase-catalyzed removal of phenols from a petroleum refinery wastewater. *Water Science and Technology* **43**(2): 253-260.

Wagner, M. and Nicell, J. A. (2001b). Treatment of a foul condensate from Kraft pulping with horseradish peroxidase and hydrogen peroxide. *Water Research* **35**(2): 485-495.

Welinder, K. G. (1976). Covalent structure of the glycoprotein horseradish peroxidase (EC 1.11.1.7). *FEBS Letters* **72**(1): 19-23.

Welinder, K. G. (1992). Superfamily of plant, fungal and bacterial peroxidases. *Current Opinion in Structural Biology* **2**: 388-393.

Wright, H. and Nicell, J. A. (1999). Characterization of soybean peroxidase for the treatment of aqueous phenol. *Bioresource Technology* **70**(1): 69-79.

Yu, J., Taylor, K. E., Zou, H., Biswas, N. and Bewtra, J. K. (1994). Phenol conversion and dimeric intermediates in horseradish peroxidase-catalyzed phenol removal from water. *Environmental Science and Technology* **28**(12): 2154-2160.

Zamocky, M., Godocikova, J., Koller, F. and Polek, B. (2001). Potential application of catalase-peroxidase from *Comamonas terrigena* N3H in the biodegradation of phenolic compounds. *Antonie van Leeuwenhoek* **79**: 109-117.

Chapter 3. Recent Developments in the Production of Extracellular Fungal Peroxidases and Laccases for Waste Treatment*

3.1. Introduction

Use of enzymes obtained from various plant and microbial sources for wastewater and solid waste treatment have been actively studied in recent years. Enzymatic processes have various advantages over conventional biological, physical and chemical treatment processes including selective removal of particular pollutants, application to xenobiotic recalcitrant compounds, high reaction rate, operation over a wide range of pH and salinity, reduction in sludge volume, and simplicity of controlling the process (Aitken, 1993; Karam and Nicell, 1997). A variety of enzymatic processes have been proposed and investigated to remove or detoxify various classes of organic and inorganic chemical pollutants (Aitken, 1993; Karam and Nicell, 1997; Nicell, 2001).

Among the enzymatic processes studied for waste treatment, peroxidase- and phenol oxidase-catalysed treatment of phenols and aromatic amines is probably the most comprehensively studied enzymatic process. Peroxidases produced by plants such as horseradish and soybean, and fungi such as white-rot fungi, as well as phenol oxidases from fungi such as mushrooms (tyrosinase) and white-rot fungi (laccase) have been studied for this process (Duran and Esposito, 2000; Nicell, 2001). Extracellular fungal peroxidases and laccases are considered to have larger potentials than plant enzymes for feasible environmental applications because of the relatively easy enzyme separation and

---

*A version of this chapter (by Ikehata, K., Buchanan, I.D. and Smith, D.W) has been submitted to the *Journal of Environmental Engineering and Science* in August 2003.

purification and the rapid growth and production in large bioreactors, which suit the bulk production of enzymes. Other than phenolic pollutants, those fungal peroxidases and laccases from white rot fungi are also effective in degrading xenobiotic pollutants including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), synthetic dyes and pesticides. Recent progress in microbiology and biotechnology also encourages the application of these fungal enzymes to waste treatment/waste detoxification processes.

Although recent studies on the enzymatic process to treat phenolic pollutants and other hazardous xenobiotics using fungal peroxidases or laccases indicate great potential, there are still a number of issues to be addressed before this and other similar enzymatic processes can be implemented at industrial scale. These issues include the development of low-cost sources of enzymes in large quantities, demonstration of the feasibility of utilising the enzymes efficiently under the conditions encountered in the actual waste treatment, and characterisation of reaction products and assessment of their impact on downstream processes, among others (Nicell, 2001).

Among these issues, reducing enzyme production costs is considered a major priority. Thus, numerous studies to increase enzyme yields and improve enzyme performance have been carried out recently in such fields as microbiology, fermentation technology and biotechnology. These efforts may provide invaluable information to advance the development and acceptance of the enzyme treatment process. Therefore, in this review, the literature dealing with recent advances in the production of extracellular fungal peroxidases and laccases for waste treatment and other environmental applications is summarised and discussed. Although the heterologous expression of recombinant fungal peroxidases and laccases has also been studied very actively in recent years

(Cassland and Jonsson, 1999; Conesa *et al.*, 2002; Hong *et al.*, 2002), this means of enzyme production will not be covered in this review.

3.2. Fungal peroxidases and fungal laccases

Peroxidases (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) are a group of haem containing oxidoreductases that catalyse the reduction of peroxide such as hydrogen peroxide (H_2O_2) and the oxidation of a variety of organic and inorganic substrates. Peroxidases of fungal origin, studied for waste treatment, include chloroperoxidase (CPO; EC 1.11.1.10), lignin peroxidase (LiP, ligninase, diarylpropane peroxidase; EC 1.11.1.14), manganese-dependent peroxidase (MnP, manganese peroxidase; EC 1.11.1.13), *Coprinus cinereus* peroxidase (CIP; = *C. macrorhizus* peroxidase; EC 1.11.1.7) and *Arthromyces ramosus* peroxidase (ARP; EC 1.11.1.7). Based on their molecular and structural similarities, these fungal peroxidases, except CPO, are classified as the class II fungal peroxidases in the plant peroxidase superfamily, which was proposed by Welinder (1992). CPO is the only exception from the plant peroxidase superfamily because it has a distinct tertiary structure and catalytic activities to other plant and fungal peroxidases (Welinder, 1992; Casella and Marchesini, 1994).

Although CPO is also a very versatile and industrially important enzyme (Pickard *et al.*, 1991; Littlechild, 1999), this enzyme will not be covered in this review because the development in CPO production process was established mostly in the 1980's (Pickard *et al.*, 1991), and virtually no report was found in the recent literature. LiP and MnP are closely related enzymes and are often produced simultaneously by white rot fungi that possess ligninolytic activities. Thus, these two enzymes will be treated as a group in this review. Because CIP and ARP are also closely related to each other and do not exhibit ligninolytic activities, these enzymes will be discussed together as well, and grouped as non-ligninolytic fungal peroxidases. A comprehensive review on the structural and catalytic properties of these fungal peroxidases is also available elsewhere (Dunford, 1999).

Laccase (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) is one of the few enzymes that was the subject of investigation as early as the end of the 19th century (Thurston, 1994). This enzyme can be further divided into two categories, plant and fungal laccases, although laccase-type phenol oxidases were also isolated from bacteria (Castro-Sowinski *et al.*, 2002) and insects (Thomas *et al.*, 1989). Laccases have multiple copper atoms at their active sites and utilise molecular oxygen as an oxidant for the oxidation of varieties of phenols and other aromatic compounds to corresponding reactive quinones (Solomon *et al.*, 1996). Comprehensive reviews on the structural and catalytic features of laccases are also available elsewhere (Thurston, 1994; Solomon *et al.*, 1996).

Recent progress in the development of the production processes for each group of enzyme are outlined below with a brief summary of the historical background, catalytic properties of the enzyme, as well as its potential application to waste treatment. More detailed reviews in the potential environmental applications of enzymes, including fungal enzymes discussed here, are also available elsewhere (Aitken, 1993; Karam and Nicell, 1997; Duran and Esposito, 2000; Nicell, 2001).

3.3. Lignin and manganese-dependent peroxidases (LiP and MnP)

3.3.1. Background

Ligninolytic class II fungal peroxidases including LiP and MnP were discovered and purified from extracellular culture medium of a basidiomycete *Phanerochaete chrysosporium* in the mid-1980's (Tien and Kirk, 1983; Kuwahara *et al.*, 1984; Tien and Kirk, 1984; Huynh and Crawford, 1985; Paszczynski *et al.*, 1985). Shortly after the discovery of the *P. chrysosporium* LiP and MnP, the production of these enzymes by many other white rot fungi were demonstrated, such as *Trametes* (*Coriolus* or *Polyporus*) *versicolor* (Dodson *et al.*, 1987), *Chrysonila sitophila* (Duran *et al.*, 1987a; Duran *et al.*, 1987b), and *Phlebia radiata* (Niku-Paavola *et al.*, 1988), among others. LiP and MnP from *P. chrysosporium* have been extensively studied as a model for fungal lignin degradation (Glenn *et al.*, 1983; Gold *et al.*, 1984; Kuwahara *et al.*, 1984; Tien and Kirk, 1984; Kirk *et al.*, 1986; Umezawa and Higuchi, 1989). Along with laccase and H_2O_2 producing oxidases such as aryl-alcohol oxidase, these ligninolytic peroxidases are responsible for the strong wood decomposition ability of the white rot fungi (Pelaez *et al.*, 1995; Martinez, 2002).

3.3.1.1. Catalytic activities of LiP and MnP

In addition to the classical peroxidase reactions in which phenolic compounds are oxidised, both LiP and MnP are capable of catalysing the degradation of lignin and a variety of non-phenolic lignin model compounds in the presence of H_2O_2, but through different mechanisms. The characteristic reactions catalysed by LiP include nonstereospecific C_α-C_β cleavage and β-*O*-4 cleavage in lignin model dimers, aromatic ring opening, oxidation of benzyl alcohols such as veratryl alcohol to corresponding aldehydes or ketones and hydroxylation of benzylic methylene groups (Tien and Kirk, 1984; Leisola *et al.*, 1985; Kirk *et al.*, 1986; Umezawa and Higuchi, 1989). Although it is generally accepted that the major role of LiP in lignin metabolism by white rot fungi is depolymerisation through the reactions listed above, this enzyme also catalyses the polymerisation of alkali lignin in the presence of veratryl alcohol *in vitro* (Haemmerli *et al.*, 1986a). Veratryl alcohol, a secondary metabolite of white rot fungi, also acts as a mediator between LiP and the polymeric substances that are less accessible to the enzyme (Harvey *et al.*, 1986). It also serves as a reducing agent of LiP compound II, an active form of the enzyme susceptible to attack and inactivation by H_2O_2, within the catalytic cycle of LiP (Harvey *et al.*, 1989).

As the name suggests, MnP requires H_2O_2 as well as divalent manganese cation, Mn(II), for its activity. The enzyme catalyses the oxidation of Mn(II) to Mn(III) that in turn oxidises various lignin related organic compounds, including vanillylacetone, 2,6-dimethyloxyphenol, curcumin, syringic acid, guaiacol, syringaldazine, divanillylacetone and coniferyl alcohol, as well as organic dyes such as Poly R, Poly B and phenol red (Paszczynski *et al.*, 1985; Glenn *et al.*, 1986; Paszczynski *et al.*, 1986). It is known that MnP does not catalyse the oxidation of veratryl alcohol. Glenn *et al.* (1986) noted that the complex of Mn(III) with α-hydroxy acid, such as lactate, worked as a diffusive intermediate in the oxidative reactions. In the absence of exogenous H_2O_2, MnP also has an oxidase activity against NADPH, GSH, dithiothreitol and dihydroxymaleic acid, forming H_2O_2 at the expense of oxygen (Paszczynski *et al.*, 1986). However, the H_2O_2 generation by MnP is not likely the sole source of peroxide in the fungal metabolism but other enzymes such as aryl-alcohol oxidase are also involved. Recently, Mn-independent peroxidases or so called MnP-LiP hybrid isozymes were discovered from the cultures of some white-rot fungi such as *Pleurotus ostreatus* (Sarkar *et al.*, 1997), *Bjerkandera* sp. (Mester and Field, 1998) and *P. eryngii* (Ruiz-Duenas *et al.*, 1999a). These enzymes catalyse the oxidation of various phenols and aromatic amines such as 2,6-dimethoxyphenol, guaiacol, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and *o*- and *p*-anisidine, as well as non-phenolic substrates such as veratryl alcohol and 1,4-dimethoxybenzene in the absence of manganese ion. It was suggested that these Mn-independent peroxidases might have potential applications in degradation of xenobiotics such as PAHs (Wang *et al.*, 2002). A detailed review of the molecular biology and structural and catalytic properties of these ligninolytic peroxidases, including LiP, MnP and Mn-independent peroxidase, was published recently (Martinez, 2002).

3.3.1.2. Application of LiP and MnP

Although the studies on the ligninolytic peroxidases were first motivated by their industrial applications in pulp and paper industries such as biochemical pulping and decolourisation of bleach plant effluent (Eaton *et al.*, 1980; Jurasek and Paice, 1986; Archibald *et al.*, 1990; Higuchi, 1990; Dezotti *et al.*, 1995; Bajpai, 1999), numerous reports were published in recent years on the use of these enzymes for the degradation of xenobiotic compounds such as PAHs (Haemmerli *et al.*, 1986b), chlorinated phenols (Hammel and Tardone, 1988; Mileski *et al.*, 1988), dioxins (Hammel *et al.*, 1986; Valli *et al.*, 1992), bisphenol A (Hirano *et al.*, 2000), and synthetic dyes (Young and Yu, 1997; Ferreira *et al.*, 2000).

A few studies on the removal of phenolic compounds using LiP and MnP though free radical coupling and polymerisation, rather than degradation, are also reported.

Aitken *et al.* (1989) investigated LiP from *P. chrysosporium* to remove various phenolic compounds from their aqueous solutions using veratryl alcohol as a co-catalyst. More recently, Tsutsumi *et al.* (2001) investigated the removal of bisphenol A and nonylphenol, and the associated estrogenic activities using MnP from *P. chrysosporium* by this manner.

3.3.2. Production of LiP and MnP

3.3.2.1. Earlier developments

Since the discovery of LiP and MnP in the cultures of the white rot fungus *P. chrysosporium*, a number of investigations have been conducted on the production of these enzymes by this fungus for their industrial and environmental applications. Earlier studies revealed that the production of ligninolytic enzymes by *P. chrysosporium* was facilitated under the conditions in which nutrients, including carbon, sulphur and, especially, nitrogen, were limited (Kirk *et al.*, 1978; Jeffries *et al.*, 1981), and was suppressed by the agitation of submerged cultures (Kirk *et al.*, 1978; Ulmer *et al.*, 1983; Faison and Kirk, 1985). These culture conditions would likely hamper the large-scale production of LiP and MnP by *P. chrysosporium* because of the limited oxygen transfer and low nutrient availability at the time of enzyme production. Several attempts to improve the LiP production by *P. chrysosporium* have been documented. These include the addition of veratryl alcohol or veratraldehyde to agitated culture media (Leisola and Fiechter, 1985) and the isolation and selection of the wild-type *P. chrysosporium* strains (Buswell *et al.*, 1984; Roch *et al.*, 1989) as well as of the mutant strains obtained through UV mutagenesis (Tien and Myer, 1990) that are capable of producing LiP under nutrient-rich culture conditions. It is generally accepted that the amount of MnP produced by *P. chrysosporium* and other white rot fungi increases by the addition of Mn(II) to the culture media (Hamman *et al.*, 1999). Although it was reported that the LiP production by several strains of *P. chrysosporium* was inversely related to the amount of Mn(II) present in the culture medium as well as to the amount of MnP produced by the fungi (Bonnarme and Jeffries, 1990), there is a report showing that the manganese addition exhibited an enhancement of the production of LiP by one *P. chrysosporium* mutant (Orth *et al.*, 1991).

3.3.2.2. Screening of fungi

As many of the investigators noted, *P. chrysosporium* may not be the best fungal species for the large-scale production of ligninolytic enzymes because of the existing limitations mentioned previously. Therefore, a number of screening studies of fungi, mostly of white rot basidiomycetes but also of other classes of fungi were conducted to discover better LiP or MnP producing fungi during the last two decades.

Waldner *et al.* (1988) compared the ligninolytic activity and peroxidase production of six white rot fungi to that of *P. chrysosporium*. In addition to *P. chrysosporium*, LiP activity was found in the culture filtrates of *Coriolus* (*Trametes*) *versicolor* and *Chrysosporium pruinosum*. Although LiP was not detected, *B. adusta* degraded lignin at a fast rate in agitated cultures. The authors detected a novel Mn(II)-independent peroxidase in the cultures of *B. adusta*. Kimura *et al.* (1990) performed a screening experiment using the *P. chrysosporium* LiP gene as a DNA probe and found LiP genes in white rot fungi *B. adusta* and *Coriolus consors*. Nerud *et al.* (1991) evaluated seven white-rot fungi and found that *Trametes gibbosa* and *T. hirsuta* produced LiP, MnP and laccase, and that *Pycnoporus cinnabarinus*, *Coriolopsis polyzona*, *Stereum hirsutum*, *Dichomitus squalens* and *Ganoderma valesiacum* produced MnP and laccase.

De Jong *et al.* (1992) tested the ligninolytic activity of sixty-seven basidiomycetes, which were isolated with a selective medium containing hemp stem wood, guaiacol and benomyl, using Poly R-478 decolourisation and ethene production from α-keto-γ-methylthiolbutyric acid (KTBA) as indicators of the activity. They also measured the enzyme activities of LiP, MnP, laccase, veratryl alcohol oxidase and glyoxal oxidase. They found that most of the Poly R-478 decolourising fungi tested produced MnP, but LiP was only found in *P. chrysosporium*.

Orth *et al.* (1993) demonstrated the ubiquity of ligninolytic peroxidases in wood-degrading fungi, which had not been evaluated previously. These included *Cyathus stercoreus*, *Dichomitus squalens*, *Ganoderma lucidum*, *Grifola frondosa*, *Lentinula edodes*, *Perenniporia medulla-panis*, *Pleurotus sapidus*, *P. eryngii*, *P. pulmonarius*, *P. (T.) versicolor* and *Trametes cingulata*. Both MnP and LiP were produced in substantially variable levels among the fungal species, and the production of the latter enzyme was only confirmed by immunoblotting tests. Pelaez *et al.* (1995) expanded the screening for ligninolytic activity to classes of basidiomycetes other than Aphyllophorales (containing most of white rot fungi), including Agaricales, Dacrymycetales, Tremellales, Auriculariales and Nidulariales. The authors found that 50%, 40% and 29% of the fungi tested (90 strains of 68 species) from the different classes of the basidiomycetes produced laccase, aryl-alcohol oxidase and MnP, respectively.

Numerous newly isolated white-rot fungi including such strains as *Trametes* spp. (Levin and Forchiassin, 2001; Tekere *et al.*, 2001; Nyanhongo *et al.*, 2002a), *Coriolus* sp. (Koroleva *et al.*, 2002b) and *Pleurotus* spp. (Eichlerova *et al.*, 2000; Ha *et al.*, 2001) that have pronounced ability to produce ligninolytic peroxidases have been introduced during the past several years. Other notable fungi recently discovered to produce either LiP or MnP are marine ascomycetes *Sordaria fimicola* and *Halosarpheia ratnagiriensis* (MnP and laccase) (Raghukumar, 2000), a marine basidiomycete *Flavodon flavus* (LiP, MnP and laccase) (Raghukumar *et al.*, 1999; Raghukumar, 2000), litter-decomposing basidiomycetes *Collybia dryophila*, *Agrocybe praecox* and *Stropharia coronilla* (MnP) (Steffen *et al.*, 2002a; 2002b), and yeasts *Debaryomyces polymorphus* and *Candida tropicalis* (MnP) (Yang *et al.*, 2003). It is also known that some actinomycete bacteria such as *Streptomyces viridosporus* have substantial lignin-degrading activity and produce extracellular LiP (Ramachandra *et al.*, 1988; Zimmermann, 1990). The fungal species evaluated recently for the production of LiP and MnP are listed in Table 3.1.

3.3.2.3. Production optimisation

After some successful screening tests, optimisation of the LiP and/or MnP production was extensively explored with the selected organisms. The fungal species most often employed include *P. chrysosporium*, *Bjerkandera* spp. (such as *B. adusta*) and *Pleurotus* spp. (such as *P. ostreatus*, *P. eryngii*). In addition to the shaken flask cultures and conventional stirred-tank bioreactors in which fungi normally grow in liquid media, solid-state or solid-substrate fermentation has been actively evaluated in the recent years (Table 3.2). This approach encourages both the enhancement of enzyme productivity through immobilisation on a solid substrate and the reduction in cost for growth substrates of fungus because agricultural or industrial waste material can be used as cheap substrates (Pandey *et al.*, 1999). Some of the recent developments for each group of fungal species are discussed below.

Table 3.1. Some fungi recently studied for the production of extracellular peroxidase and laccase.

Fungus	Type	Enzyme	Reference
Agaricus sp.	Wood decaying basidiomycete	Laccase	Kaluskar *et al.* (1999)
Agrocybe praecox	Litter-decomposing basidiomycete	MnP	Steffen *et al.* (2002b)
Arthromyces ramosus	Deuteromycete (soil fungus)	ARP	Yao *et al.* (1995)
Bjerkandera adusta (*Bjerkandera* sp.)	White rot	LiP, MnP	Mester and Field (1997), Nakamura *et al.* (1999), Pickard *et al.* (1999b), Moreira *et al.* (2000a; 2000b), Wang *et al.* (2001)
Botryosphaeria sp.	Ascomycete	Laccase	Vasconcelos *et al.* (2000), Dekker and Barbosa (2001), Dekker *et al.* (2001)
Candida tropicalis	Yeast	MnP	Yang *et al.* (2003)
Cerrena maxima	White rot	Laccase	Koroleva *et al.* (2002a)
Chalara paradoxa	Deuteromycete	Laccase	Robles *et al.* (2000; 2002)
Clitocybula dusenii	Agaric white rot	MnP	Nuske *et al.* (2002)
Collybia dryophila	Litter-decomposing basidiomycete	MnP	Steffen *et al.* (2002a)
Coniothyrium minitans	Soil fungus	Laccase	Dahiya *et al.* (1998)
Coprinus cinereus	Inky-cap mushroom	CIP	Sakurai *et al.* (2002), Ikehata and Buchanan (2002)
C. lagopus	Inky-cap mushroom	Peroxidase	Ikehata and Buchanan (2002)
Coprinus sp.	Inky-cap mushroom	Peroxidase	Ikehata *et al.* (2003b)
Coriolopsis gallica	White rot	Laccase	Pickard *et al.* (1999b)
C. rigida	White rot	Laccase	Saparrat *et al.* (2002)

Table 3.1. Some fungi recently studied for the production of extracellular peroxidase and laccase (continued).

Fungus	Type	Enzyme	Reference
Cyathus bulleri	Wood degrading basidiomycete	Laccase	Dhawan and Kuhad (2002), Dhawan *et al.* (2003)
C. stercoreus	Wood degrading basidiomycete	Laccase	Sethuraman *et al.* (1999), Dhawan and Kuhad (2002)
Debaryomyces polymorphus	Yeast	MnP	Yang *et al.* (2003)
Flavodon flavus	Marine basidiomycete	LiP, MnP, laccase	Raghukumar *et al.* (1999)
Fomes sclerodermeus	White rot	MnP, laccase	Papinutti *et al.* (2003)
Ganoderma lucidum	White rot	LiP, MnP, laccase	D'Souza *et al.* (1999)
Lentinula edodes	White rot	MnP, laccase	Hatvani and Mecs (2001; 2002), Morais *et al.* (2001a)
Marasmius quercophilus	White rot	Laccase	Klonowska *et al.* (2001)
Nematoloma frowardii	Agaric white rot	LiP, MnP, laccase	Hofrichter *et al.* (1999), Nuske *et al.* (2002)
Paecilomyces inflatus	Deuteromycete	Laccase	Kluczek-Turpeinen *et al.* (2003)
Panus tigrinus	White rot	MnP	Fenice *et al.* (2003)
Phanerochaete chrysosporium	White rot	LiP, MnP	Herpoel *et al.* (1999), Manimekalai and Swaminathan (1999), Rodriguez *et al.* (1999), Annadurai *et al.* (2000), Rivela *et al.* (2000), Couto *et al.* (2001; 2002a; 2002c), Dominguez *et al.* (2001a; 2001b), Cabaleiro *et al.* (2002)

Table 3.1. Some fungi recently studied for the production of extracellular peroxidase and laccase (continued).

Fungus	Type	Enzyme	Reference
Phanerochaete chrysosporium (continued)	White rot	LiP, MnP	Gill and Arora (2003), Govender *et al.* (2003), Moldes *et al.* (2003), Grgic and Perdih (2003)
P. flavido-alba	White rot	LiP, MnP	Hamman *et al.* (1999)
Phlebia floridensis, *P. brevispora*, *P. radiata*, *P. fascicularia*	White rot	Laccase	Arora and Gill (2001), Arora and Rampal (2002)
Pleurotus eryngii	White rot	MnP	Munoz *et al.* (1997b; 1997a)
P. florida	White rot	Laccase	Das *et al.* (1999)
P. ostreatus	White rot	MnP, laccase	Eichlerova and Homolka (1999), Medeiros *et al.* (1999), Novotny *et al.* (1999), Eichlerova *et al.* (2000), Giardina *et al.* (2000), Ha *et al.* (2001), Morais *et al.* (2001b), Reddy *et al.* (2003)
P. pulmonarius	White rot	Laccase	De Souza *et al.* (2002)
P. sajor-caju	White rot	MnP, laccase	Lo *et al.* (2001), Reddy *et al.* (2003)
Pycnoporus cinnabarinus	White rot	Laccase	Herpoel *et al.* (2000), Lomascolo *et al.* (2003)
P. sanguineus	White rot	Laccase	Pointing *et al.* (2000), Lomascolo *et al.* (2002)
Rigidoporus lignosus	White rot	Laccase	Cambria *et al.* (2000)
Stropharia coronilla	Litter-decomposing basidiomycete	MnP	Steffen *et al.* (2002b)

Table 3.1. Some fungi recently studied for the production of extracellular peroxidase and laccase (continued).

Fungus	Type	Enzyme	Reference
Trametes (*Coriolus* or *Polyporus*) *versicolor*	White rot	LiP, MnP, laccase	Lee *et al.* (1999), Ullah *et al.* (2000), Kahraman and Gurdal (2002), Lorenzo *et al.* (2002), Mougin *et al.* (2002), Gill and Arora (2003), Couto *et al.* (2002d; 2003)
T. cingulata, *T. elegans*, *T. pocas*	White rot	MnP, laccase	Tekere *et al.* (2001)
T. hirsuta (*hirsutus*)	White rot	LiP, MnP, laccase	Koroljova-Skorobogat'ko *et al.* (1998), Koroleva *et al.* (2002a; 2002b), Rosales *et al.* (2002)
T. modesta	White rot	Laccase	Nyanhongo *et al.* (2002a)
T. multicolour	White rot	Laccase	Hess *et al.* (2002)
T. pubescens	White rot	Laccase	Galhaup and Haltrich (2001), Galhaup *et al.* (2002)
T. trogii	White rot	LiP, MnP, laccase	Levin and Forchiassin (2001), Levin *et al.* (2002)
Trametes sp.	White rot	Laccase	Jang *et al.* (2002)
Trichophyton rubrum	Wood degrading fungus	Laccase	Jung *et al.* (2002)

3.3.2.4. Phanerochaete *spp.*

Because *P. chrysosporium* is definitely the most-well studied fungus for ligninolytic peroxidase production, the recent studies with this fungus have been focused mostly on bioreactor configuration for scaling up and continuous production, and the use of statistical methods for the process optimisation. Herpoel *et al.* (1999) reported MnP

Table 3.2. Solid substrates and other waste products recently evaluated for the production of LiP, MnP and laccase.

Substrate	Fungus	Enzyme	Reference
Banana waste	*P. ostreatus*, *P. sajor-caju*	LiP (MnP), laccase	Reddy *et al.* (2003)
Barley bran	*T. versicolor*	Laccase	Lorenzo *et al.* (2002), Couto *et al.* (2002d; 2003)
Barley straw	*T. versicolor*	Laccase	Couto *et al.* (2002d)
Grape stalk, grape seed	*T. versicolor*	Laccase	Lorenzo *et al.* (2002)
Molasses wastewater (vinasse) with cotton stalk	*C.* (*T.*) *versicolor*, *Funlia trogii*	Laccase	Kahraman and Gurdal (2002)
Olive mill wastewater	*P. tigrinus*	MnP, laccase	Fenice *et al.* (2003)
Potato peeling with barley bran	*T. hirsuta*	Laccase	Rosales *et al.* (2002)
Rice bran	*B. adusta*	MnP	Wang *et al.* (2001)
Wheat bran	*C. gallica*	Laccase	Pickard *et al.* (1999b)
	F. sclerodermeus	MnP, laccase	Papinutti *et al.* (2003)
	P. pulmonarius	Laccase	De Souza *et al.* (2002)
Wheat bran, wheat husk	*C.* (*T.*) *versicolor*	Laccase	Ullah *et al.* (2000)
Wheat straw	*L. edodes*	Laccase	Hatvani and Mecs (2002)
	P. ostreatus	Laccase	Morais *et al.* (2001b)
	T. versicolor	Laccase	Couto *et al.* (2002d)
Wood sawdust	*P. ostreatus*	MnP	Giardina (2000)
Wood shaving	*T. versicolor*	Laccase	Couto *et al.* (2002d)

production by *P. chrysosporium* I-1512 in an airlift bioreactor with a working volume of 100 L. High MnP production of 6600 U L^{-1} was achieved in this scaled-up configuration after 150 hours of operation. Manimekalai and Swaminathan (1999) reported an optimised culture condition for LiP production in batch cultures of *P. chrysosporium* ATCC 24725 using a 2^3 full factorial central composite design with response surface methodology.

A series of semi-solid-state and solid-state cultivation studies of *P. chrysosporium* BKM-F-1767 (ATCC 24725) was carried out for the purpose of stable continuous production of LiP and MnP with different bioreactor configurations. As support media, inert plastic carriers, such as cubes of polyurethane foam and cubes of nylon sponge, and chopped corncob were evaluated for immobilization of the fungus (Rodriguez *et al.*, 1999b; Couto *et al.*, 2000). Of these solid media, the authors found nylon sponge supported the highest MnP activity of 1593 U L^{-1}. Various bioreactor configurations were tested for batch or continuous production of LiP and MnP by *P. chrysosporium* including an airlift bioreactor (Dominguez *et al.*, 2001a), a rotating drum bioreactor (Dominguez *et al.*, 2001b), an immersion bioreactor (Rivela *et al.*, 2000; Couto *et al.*, 2002b) and a fixed-bed bioreactor (Couto *et al.*, 2002c) using nylon sponge cubes for fungal immobilisation. Couto *et al.* (2002a) noted that continuous operation of the immersion bioreactor showed a lower MnP productivity, but a higher LiP productivity compared to batch operation, and that higher aeration rate significantly increased the LiP productivity. Couto *et al.* (2002c) also noted that the addition of 1 mM veratryl alcohol and 1 mM tryptophan, no Mn^{2+} addition, low glucose addition rate and aeration facilitated the production of LiP up to 700 U L^{-1} in a continuously operated fixed-bed bioreactor.

Recently, a membrane gradostat reactor for continuous production of MnP by *P. chrysosporium* ME446 was reported (Govender *et al.*, 2003). The fungus was immobilised on ultrafiltration capillary membranes, which demonstrated reproducible continuous production of MnP and could be scaled up without deteriorating enzyme productivity. Other than bioreactor configurations, Grgic and Perdih (2003) recently demonstrated that the addition of poly(ethylene glycol) (PEG) and some other polyoxyalkanes had stimulating effects on the LiP production by *P. chrysosporium* MZKI B-223 (ATCC 24725) in shaken cultures. Of the substances tested, the authors found that PEG with an average molecular weight of 10 000 kDa showed the best stimulation effect (up to 50 times higher than in the control culture) on the LiP production by this fungus.

3.3.2.5. Bjerkandera *spp.*

The production of ligninolytic peroxidases in cultures of *Bjerkandera* species was first demonstrated in several early screening experiments (Waldner *et al.*, 1988; Kimura *et al.*, 1990; De Jong *et al.*, 1992). It was shown that MnP and laccases, as well as Mn-independent peroxidases were produced by *Bjerkandera* spp. Although LiP was not apparently produced by *Bjerkandera* spp. during the early investigations, its production was induced by nitrogen sufficient culture conditions, which suppressed the LiP production by *P. chrysosporium* (Kaal *et al.*, 1993). Mester *et al.* (1996) further revealed that the ligninolytic activity of *Bjerkandera* sp. BOS55 was initiated by the cessation of growth and triggered by carbon limitation. The MnP production by the same strain of *Bjerkandera* sp. was increased up to 1250 U L^{-1} by optimising cultivation conditions including Mn concentration, pH, temperature and the addition of organic acids such as glycolate, malonate, glucuronate, gluconate and 2-hydroxybutyrate (Mester and Field, 1997). Nakamura *et al.* (1999) reported improved production of MnP, LiP and laccase by *B. adusta* IFO 4983 immobilised on polyurethane foam. Moreira and co-workers studied the MnP production by *Bjerkandera* sp. BOS55 in batch shaker flasks (Moreira *et al.*, 2000a) as well as stirred fermentors with 2-, 10- and 50-L of capacities (Moreira *et al.*, 2000b). The authors showed that the scaling up did not deteriorate the enzyme production and high level of MnP production (480 U L^{-1}) was achieved in 50-L fermentors. Unlike other reports, the immobilisation of *Bjerkandera* sp. BOS55 on polyurethane foam did not improve MnP production but rather suppressed it. Wang *et al.* (2001) showed *B. adusta* UAMH 7308 and UAMH 8258 produced more MnP than *Bjerkandera* sp. (*B. adusta*) BOS55 (ATCC 90940) in cereal bran media, and achieved 5500 U L^{-1} of MnP by *B. adusta* UAMH 7308 in 100-mL rice bran medium under an optimal condition. The authors also demonstrated large production of MnP (3500 U L^{-1} and 2500 U L^{-1} by UAMH 8258 and UAMH 7308, respectively) in 10-L stirred tank reactors.

3.3.2.6. Pleurotus *spp.*

Pleurotus species are another class of actively studied white-rot fungi for the production of ligninolytic peroxidases. Many of *Pleurotus* spp., such as *P. ostreatus* and *P. pulmonarius*, form edible mushrooms and have been studied to produce the mushrooms in conjunction with bioremediation and waste conversion (Velazquez-Cedeno *et al.*, 2002). It was recently reported that the ligninolytic peroxidases produced by one of *Pleurotus* spp., *P. eryngii*, had very unique catalytic activities compared with conventional LiP and MnP produced by *P. chrysosporium* (Caramelo *et al.*, 1999; Ruiz-Duenas *et al.*, 1999a; Ruiz-Duenas *et al.*, 1999b; Gomez-Toribio *et al.*, 2001). Giardina *et al.* (2000) studied the solid-state fermentation of *P. ostreatus* for the production of MnP with wood sawdust as a growth substrate. The authors examined three different wood sawdusts (poplar, fir and chestnut) and found that poplar sawdust was the better support for both growth and MnP production by *P. ostreatus*. Ha *et al.* (2001)

showed that the agitation, which enhanced oxygen transfer, greatly improved the MnP production by *P. ostreatus* in a liquid medium. More recently, Reddy *et al.* (2003) demonstrated the utilisation of banana waste for the production of laccase and LiP (or MnP) by solid substrate fermentation of two *Pleurotus* species, including *P. ostreatus* and *P. sajor-caju*. The authors suggested that the use of cheap and abundant banana waste would be a good strategy to produce industrially important ligninolytic enzymes and to reduce the amounts of agricultural waste by solid substrate fermentation.

3.3.2.7. *Other white-rot fungi*

Some other fungi were also investigated for LiP or MnP production recently. Nuske *et al.* (2002) reported a large-scale production of MnP by two agaric white rot fungi, *Nematoloma* (*Hypholoma*) *frowardii* strain b19 and *Clitocybula dusenii* strain b11, in 5-litre stirred-tank bioreactors with maximum MnP activities of 2014 and 1177 U L^{-1}, respectively. They found that scale-up to 30- and 300-litre stirred-tank bioreactors did not show significant loss in productivity of MnP by *N. frowardii*, and that sodium acetate was preferred over glucose as a carbon source by the fungus. Papinutti *et al.* (2003) reported the production of laccase and MnP by a white rot fungus *Fomes sclerodermeus* BAFC 2752 using wheat bran as a growth substrate, and obtained a maximum MnP activity of 6.3 U g^{-1} of dry substrate. Fenice *et al.* (2003) studied the use of an olive mill wastewater-based medium for the production of laccase and MnP by *Panus tigrinus* CBS 577.79. The authors found that a maximum MnP activity of 410 U L^{-1} in an airlift bioreactor, and that the use of olive mill wastewater-based medium enhanced the productivity of MnP as well as that of laccase.

3.4. Non-ligninolytic fungal peroxidases

3.4.1. Background

The newest varieties of class II fungal peroxidases with no ligninolytic activities were discovered in the mid-1980s. These include ARP from an imperfect fungus (deuteromycete) *Arthromyces ramosus* isolated from soil, and CIP from an inky cap basidiomycete *Coprinus cinereus* (Shinmen *et al.*, 1986; Morita *et al.*, 1988). There is another variety of fungal peroxidase called CMP obtained from *C. macrorhizus*. However, this fungus is recognised as the same species as *C. cinereus* (Orton and Watling, 1979), and it was suggested that these two *Coprinus* peroxidases were identical. Indeed, the analyses of amino acid sequences, three-dimensional molecular structures and catalytic activities of the three fungal peroxidases (CIP, CMP and ARP) have revealed that these enzymes are identical except for a slight difference in glycosylation, which has no influence on the reaction rate of the enzyme (Kjalke *et al.*, 1992; Baunsgaard *et al.*, 1993; Sawai-Hatanaka *et al.*, 1995).

3.4.1.1. Catalytic activity of non-ligninolytic fungal peroxidases

Although ARP and CIP have very similar catalytic activities to HRP, a classical plant peroxidase, there is less similarity in overall amino acid sequences between the genes encoding these plant and fungal enzymes (Baunsgaard *et al.*, 1993). On the other hand, whereas the amino acid sequences of ARP and CIP have shown better agreement with those of LiP and MnP than with those of HRP, ARP and CIP exhibit no ligninolytic activities. Some significant structural differences at the active sites of the two types of fungal peroxidases were observed by X-ray crystallography (Kunishima *et al.*, 1994;

Petersen *et al.*, 1994) that might account for the differences in substrate specificity. A comprehensive review on the structural and functional features as well as the potential applications of the non-ligninolytic fungal peroxidases is available elsewhere (Nakayama and Amachi, 1999).

3.4.1.2. Application of non-ligninolytic fungal peroxidases

Non-ligninolytic fungal peroxidases were primarily investigated as alternative enzymes to HRP in clinical applications such as luminol-mediated glucose and cholesterol assays because of the high chemiluminescence intensity obtained by the ARP-and CIP-catalysed oxidation of luminol (Akimoto *et al.*, 1990; Kim *et al.*, 1991). More recently, their application to the removal of phenolic compounds from wastewater has also drawn much attention because of the catalytic similarity of these enzymes to HRP, as well as the potentials for the large-scale production. It was shown that various phenolic compounds could be removed from their aqueous solutions using ARP or CIP (CMP) (Al-Kassim *et al.*, 1993; Al-Kassim *et al.*, 1994; Kauffmann *et al.*, 1999; Sakurai *et al.*, 2001). A few studies on the application of these enzymes to the treatment of real wastewater were also reported (Ibrahim *et al.*, 2001; Ikehata *et al.*, 2003a).

3.4.2. Production of non-ligninolytic fungal peroxidases

Relatively little information has been published on the production non-ligninolytic fungal peroxidases to date (Table 3.1). Tsujimura *et al.* (1994) reported the successful scale-up production of ARP by *A. ramosus* strain 60-117 in 1-kL and 10-kL batch jar fermentors. These authors achieved 48 U mL^{-1} of ARP in a 10-kL fermentor after 110 hours of operation. Yao *et al.* (1995) studied continuous production of ARP by *A.*

ramosus strain 60-117 with mycelium recycle by filtration and achieved better enzyme production rate compared to those by fed-batch and continuous fermentation without mycelium recycle. The authors noted that the high agitation rate to maintain the dissolved oxygen level, as well as an excessively high mycelial concentration were likely to deteriorate the peroxidase production by the fungus. Sakurai *et al.* (2002) investigated the production of CIP in repeated batch cultures of *C. cinereus* IFO 30628 grown on rotating disk contactors. The authors found that a higher production rate and activity of peroxidase could be achieved in the attached growth system than was in the shaker flasks and jar fermentors employed previously.

Ikehata and Buchanan (2002) performed a series of screening experiments to identify fungi that produce non-ligninolytic peroxidase in large quantities. The authors compared twenty-five strains of *Coprinus* species for their peroxidase production and found three promising strains of *C. cinereus* including UAMH 4103, UAMH 7904 and IFO 30116. The authors also noted that several *Coprinus* species, including non-identified and identified ones such as *C. lagopus*, were also capable of producing peroxidase. Ikehata *et al.* (2003b) also evaluated several newly isolated *Coprinus* species from urea treated soil and found that one strain, *Coprinus* sp. UAMH 10067, produced a peroxidase exhibiting higher thermal stability than CIP.

3.5. Fungal laccases

3.5.1. Background

Fungal laccases are isolated and purified from the cultures of numerous fungal species and have been studied extensively for their industrial and environmental applications. Fungal laccases are implicated in several physiological roles, including morphogenesis (De Vries *et al.*, 1986; Zhao and Kwan, 1999), plant pathogenesis (Kwon and Anderson, 2001), interspecific fungal interactions (Score *et al.*, 1997) and ligninolysis. Although the latter aspect is a very complicated issue, understanding its mechanism is extremely important because the current environmental applications of fungal laccases, such as decolourisation of phenolic effluents and xenobiotics degradation and detoxification, are based on the strong oxidative ability of this class of enzyme observed during the lignin degradation. Therefore, this issue will be discussed briefly below.

3.5.1.1. Catalytic activities of laccase

Although the contribution of laccase to lignin degradation by white rot fungi such as *T. versicolor* had long been speculated (Kirk *et al.*, 1968), its role in ligninolysis was less clear than those of LiP and MnP, partly because its low redox potential did not seem to be suitable for the oxidation of non-phenolic lignin structures (Eggert *et al.*, 1997). Furthermore, both polymerisation (Leonowicz *et al.*, 1984) and depolymerisation (Kirk *et al.*, 1968; Kawai *et al.*, 1988) of lignin model compounds were reported *in vitro* with fungal laccase from *T. versicolor* and *Rhizoctonia praticola*, which made this issue more complicated. Subsequently, Leonowicz *et al.* (1985) demonstrated that depolymerisation of lignin model compounds occurred when a high molecular weight fraction of the compounds was treated with *T. versicolor* laccase, whereas polymerisation tended to occur when a low molecular weight fraction of the compounds was treated. Szklarz and Leonowicz (1986) reported that the addition of glucose oxidase to the reaction medium enhanced the decomposition of high molecular weight lignin model compounds by laccase. Glucose oxidase presumably reduces quinones produced by laccase-catalysed reactions, which prevents the re-polymerisation of depolymerised lignin. A similar effect of glucose oxidase was observed in the *Rigidoporus lignosus* MnP-laccase system, in which glucose oxidase might be also involved in the H_2O_2 production for MnP-catalysed oxidation through glucose/glucose oxidase reactions (Galliano *et al.*, 1991). Marzullo *et al.* (1995) also demonstrated that the veratryl alcohol and veratryl alcohol oxidase produced by *P. ostreatus* were also capable of reducing synthetic quinones, quinonoids, and phenoxy radicals, and proposed the cooperative action of laccase and veratryl alcohol oxidase during the lignin degradation by this fungus. Bourbonnais and Paice (1990) discovered that the barrier of redox potential for oxidising non-phenolic lignin structures could be overcome by the addition of primary laccase substrates such as ABTS that acted as a mediator. Eggert *et al.* (1996a) found a white-rot fungus *Pycnoporus cinnabarinus*, which produces only laccase as its ligninolytic system, also produced a metabolite, 3-hydroxyanthranilate, which acted as a mediator to decompose several synthetic lignin model dimers into monomers. More recently, it was shown that laccase was involved in the formation of superoxide anion radical ($O_2^{\cdot -}$) and hydroxyl radical through the oxidation of lignin-derived hydroquinones in the presence of Fe^{3+} and a chelating agent (Guillen *et al.*, 1997; Guillen *et al.*, 2000; Saparrat *et al.*, 2002). These reactive oxygen species are considered to be involved in the strong degradative activity of laccase-producing white rot fungi as well.

In addition to the ligninolysis, typical fungal laccase catalyses the oxidation of diphenols, such as catechol and hydroquinone, as well as various monophenols and aromatic amines, to free radicals that subsequently undergo further enzymatic oxidation to quinones and their oxidative couplings (Shuttleworth and Bollag, 1986; Thurston, 1994). However, laccase does not catalyse the oxidation of tyrosine, which is a unique substrate for tyrosinase; therefore, these two enzymes can be distinguished from each other. Laccase also has its unique substrate, *N'*-bis(3,5-dimethoxy-4-hydroxybenzylidene hydrazine), also called syringaldazine (Thurston, 1994). It is also known that laccase can mediate the oxidation of non-phenolic xenobiotics such as PAHs (Collins *et al.*, 1996; Pickard *et al.*, 1999a) through the oxidation of a redox mediator, which was first introduced by Bourbonnais and Paice (1990). The applicable redox mediators include ABTS (Bourbonnais and Paice, 1990), 3-hydroxyanthranilate (Eggert *et al.*, 1996a), 1-hydroxybenzotriazole (Bourbonnais *et al.*, 1998; Srebotnik and Hammel, 2000), phenol, aniline, 4-hydroxybenzoic acid, 4-hydroxybenzyl alcohol and some non-aromatic compounds containing sulfhydryl groups such as methionine, cysteine and reduced glutathione (Johannes and Majcherczyk, 2000).

3.5.1.2. Application of fungal laccases

Similar to LiP and MnP, the use of laccase for industrial and environmental purposes was initially stimulated by its potential application to the pulp and paper industry, especially in the decolourisation of bleach plant effluents, as well as phenol removal from wastewater through enzymatic oxidation, polymerisation and precipitation (e.g. Bollag *et al.*, 1988; Roper *et al.*, 1995; Karam and Nicell, 1997). However, the oxidative degradation of xenobiotics such as PAHs using so-called laccase mediator system has received increased attentions in the recent years (e.g. Collins *et al.*, 1996; Pickard *et al.*, 1999a; Duran and Esposito, 2000). A number of studies on decolourisation of industrial dyes using crude laccase preparations (Rodriguez *et al.*, 1999a; Nyanhongo *et al.*, 2002b) as well as on removal of alkylphenols through oxidative coupling (without addition of mediators) (Tanaka *et al.*, 2000; Tanaka *et al.*, 2001) were also reported recently.

3.5.2. Production of fungal laccases

Laccase activity was detected in the cultures of a wide range of fungi, from ascomycetes to basidiomycetes, and from wood and litter decomposing fungi to ectomycorrhizal fungi (Bollag and Leonowicz, 1984; Burke and Cairney, 2002). Fungi recently studied for producing extracellular laccase are listed in Table 3.1. Among these fungi, *Trametes* species are probably the most actively investigated for the laccase production because these fungi are commonly found in many parts of the world and apparently are excellent wood decomposers in nature. Indeed, *T. versicolor*, a representative fungus in this genus, is among the first fungi, from which the production of large amounts of laccase has been reported (Fahraeus and Reinhammar, 1967), and has already been marketed by several companies, although the current prices seem still too high for bulk environmental application (Duran and Esposito, 2000). Therefore, improving the productivity and reducing the production cost are the major goals for the current studies on the laccase production. Some of the recent developments in the production of fungal laccase for environmental or industrial purposes are summarised below.

3.5.2.1. Inducers

The production of laccase is affected by several factors including the composition of growth medium, pH, carbon:nitrogen ratio, temperature, and aeration rate. It was also shown that the production of laccase by *T. versicolor* and other white-rot fungi increased, or was induced, when an aromatic compound such as veratryl alcohol, vanillic acid, 2,5-xylidine, ferulic acid, syringaldazine and guaiacol, or Cu^{2+} was added to the growth medium (e.g. Bollag and Leonowicz, 1984; Eggert *et al.*, 1996b; Collins and Dobson, 1997; Munoz *et al.*, 1997b; Koroljova-Skorobogat'ko *et al.*, 1998). Since the addition of these compounds greatly enhances the laccase production and seems a promising approach for large-scale production of this enzyme for industrial applications, many reports regarding the use of inducers have been published in recent years (Table 3.3). For example, Galhaup and Haltrich (2001) confirmed the effect of copper ($CuSO_4$) addition on the laccase producing fungus *T. pubescens* MB 89 and found that the addition of copper during the exponential growth phase of the fungus provided the maximal effect on the laccase production. A similar effect was observed in the cultures of two other *Trametes* species, *T. multicolour* MB 49, *T. trogii* BAFC 463 (Hess *et al.*, 2002; Levin *et al.*, 2002) with an optimum Cu concentration ranging from 0.5 mM to 2.0 mM.

The positive effect of aromatic inducers such as guaiacol, veratryl alcohol, *p*-hydroxybenzoic acid and 2,5-xylidine on the laccase production was confirmed in the cultures of white-rot fungi such as *Phlebia radiata* MJL-1198-Sp, *Dichomitus flavida* MTCC 145 (Arora and Gill, 2001) and *Marasmius quercophilus* C30 (Klonowska *et al.*, 2001), as well as non-white-rot fungi such as *Agaricus* sp. (Kaluskar *et al.*, 1999) and an ascomycete *Botryosphaeria* sp. (Dekker and Barbosa, 2001; Dekker *et al.*, 2001). Sethuraman *et al.* (1999) reported that the addition of 3,4-dimethoxycinnamic acid at a concentration of 0.5 mM also enhanced laccase production in the cultures of *Cyathus stercoreus* ATCC 36910. Lo *et al.* (2001) evaluated the addition of various lignin monomers on the growth of *Pleurotus sajor-caju* CMB025 and its laccase production. Among the monomers tested, the authors found that 2 mM 4-hydroxybenzaldehyde and

Table 3.3. Examples of inducers recently evaluated for laccase production.

Compound	Fungus	Reference
Biotin	*C. bulleri*	Dhawan and Kuhad (2002)
$CuSO_4$ (Cu^{2+})	*M. quercophilus*	Klonowska *et al.* (2001)
	T. pubescens	Galhaup and Haltrich (2001)
	T. multicolour	Hess *et al.* (2002)
	T. troggi	Levin *et al.* (2002)
3,4-Dimethoxycinnamic acid	*C. stercoreus*	Sethuraman *et al.* (1999)
N,N'-Dimethyl -*N*-(5-chloro-4-hydroxyphenyl)urea	*T. versicolor*	Mougin *et al.* (2002)
Ethanol	*T. versicolor*	Lee *et al.* (1999)
	Botryosphaeria sp.	Dekker *et al.* (2001)
	P. cinnabarinus	Lomascolo *et al.* (2003)
Ethidium bromide	*C. bulleri*	Dhawan *et al.* (2003)
Ferulic acid	*P. cinnabarinus*	Lomascolo *et al.* (2003)
Glycine, DL-methionine, DL-valine	*C. bulleri*	Dhawan and Kuhad (2002)
4-Hydroxybenzaldehyde	*P. sajor-caju*	Lo *et al.* (2001)
p-Hydroxybenzoic acid	*M. quercophilus*	Klonowska *et al.* (2001)
4-*n*-Nonylphenol	*T. versicolor*	Mougin *et al.* (2002)
Pyridoxine hydrochloride	*C. bulleri*	Dhawan and Kuhad (2002)
Riboflavin	*C. bulleri*	Dhawan and Kuhad (2002)
Tryptophan	*T. trogii*	Levin and Forchiassin (2001)
	C. bulleri	Dhawan and Kuhad (2002)
Tween 80	*P. sanguineus*	Pointing *et al.* (2000), Lomascolo *et al.* (2002)
Vanillin	*P. sajor-caju*	Lo *et al.* (2001)
Veratryl alcohol	*Botryosphaeria* sp.	Vasconcelos *et al.* (2000), Dekker and Barbosa (2001), Dekker *et al.* (2001)
2,5-Xylidine	*C. gallica*	Pickard *et al.* (1999b)
	P. sanguineus	Pointing *et al.* (2000), Lomascolo *et al.* (2002)
	T. versicolor	Couto *et al.* (2003)

vanillin enhanced the laccase production.

The addition of various amino acids and vitamins also showed stimulating effects on the laccase production by some fungi. Levin and Forchiassin (2001) reported the addition of tryptophan to increase laccase production in the cultures of *T. trogii* BAFC 463. Dhawan and Kuhad (2002) tested 23 amino acids and 6 vitamins for their effects on laccase production by *Cyathus bulleri* 195062 and found that DL-methionine, DL-tryptophan, glycine, DL-valine, biotin, riboflavin and pyridoxine hydrochloride showed positive effects.

Lee *et al.* (1999) reported the positive effect of ethanol, a less expensive and less toxic alternative, which increased laccase production by a factor of 20 in cultures of *T. versicolor* ATCC 20869. A similar effect of ethanol was observed in the cultures of *Botryosphaeria* sp. MAMB-5 (Dekker *et al.*, 2001), as well as those of *Pycnoporus cinnabarinus* (Lomascolo *et al.*, 2003). It was also reported that the addition of ethanol, however, reduced the laccase production in the semi-solid-state culture of *T. versicolor* CBS100.29 in which nylon sponge was used as an inert support (Maceiras *et al.*, 2001). The authors suggested that ethanol might stimulate laccase production only in cultures showing melanin formation.

In addition to the lignin-related phenolics and natural organic compounds, several xenobiotics were tested for their inducing effect. Mougin *et al.* (2002) demonstrated that the addition of 4-*n*-nonylphenol, aniline and oxidised derivatives of diquat and *N*,*N'*-dimethyl-*N*-(5-chloro-4-hydroxyphenyl)urea induced the laccase production in cultures of *T. versicolor* ATCC 32745, although these chemicals were less effective than 2,5-xylidine, a well known inducer. A surfactant, Tween 80, was also tested as an inducer for the production of laccase by *Pycnoporus sanguineus* CY788, but was also found to be less effective than 2,5-xylidine (Pointing *et al.*, 2000). More recently, ethidium bromide, a DNA intercalating agent, was used to stimulate the laccase production by *C. bulleri* Brodie 195062 (Dhawan *et al.*, 2003). The authors found that when the fungus was treated with 1.5 μg mL^{-1} of ethidium bromide, the laccase production increased by six-fold compared to control culture.

3.5.2.2. Use of waste products and solid-state fermentation

The use of natural solid substrates, especially lignocellulosic agricultural residues, as growth substrates of fungi has been enthusiastically studied for laccase production in recent years (Table 3.2). Similar to the cases of LiP and MnP, this approach is attractive because of anticipated effects on cost reduction, waste reuse and enhanced enzyme production. Various solid substrates were evaluated during the past few years. Pickard *et al.* (1999b) demonstrated that the cereal bran liquid medium supported a high production of laccase by *C. gallica* UAMH 8260. Ullah *et al.* (2000) investigated wood chips, cereal grain, wheat husk and wheat bran as substrates of *Coriolus* (*Trametes*) *versicolor* FPRL-28A and found that wheat husk and wheat bran supported the growth and laccase production of this fungus very well. The authors formulated the wheat bran into pellets with biscuit flour and yeast extract and showed that it could be used as a compact substrate for fungal immobilisation. Wheat bran was also successfully used as a solid-state medium for the laccase production by *Pleurotus pulmonarius* CCB-19 (De Souza *et al.*, 2002) and *Fomes sclerodermeus* BAFC 2752 (Papinutti *et al.*, 2003). Morais *et al.* (2001b) demonstrated that wheat straw was a better substrate of *P. ostreatus* than wheat extract for the laccase production. Similar stimulation effect of wheat straw on the laccase production was observed in the cultures of *Lentinula edodes* 610 (Hatvani and

Mecs, 2002). Hatvani and Mecs (2001) also evaluated malted barley waste from the brewing process mixed with wood chips as a solid substrate for *L. edodes* 610 in three different cultivation systems: batch solid-state, flow-through and repeated batch semi-solid-state systems. The authors found that laccase and MnP were produced in all systems, but suggested that the semi-solid state system in which fungal mycelia were grown on the brewing waste and contacted with a liquid medium that was harvested and replenished periodically, was suitable for continuous production of the enzymes. Lorenzo *et al.* (2002) reported that barley bran was a better substrate for laccase production by *T. versicolor* CBS100.29 than were grape seed and grape stalks. Kahraman and Gurdal (2002) evaluated the use of molasses wastewater (vinasse) as a natural medium with or without cotton stalk as a supplement for the laccase production by *C. versicolor* ATCC 200801 and *Funlia trogii* ATCC 200800. The authors found that vinasse medium with cotton stalk gave a better result than did synthetic media or vinasse medium alone. Rosales *et al.* (2002) evaluated the use of various food wastes including apple, orange and potato peelings with barley bran for the production of laccase by *T. hirsuta* BT 2566 and found the potato peelings with barley bran gave the highest laccase activity. More recently, Fenice *et al.* (2003) demonstrated the successful use of olive mill wastewater-based medium, which contained large amounts of recalcitrant aromatic compounds, for the production of laccase and MnP in submerged and solid-state cultures of *Panus tigrinus*.

3.5.2.3. Selective breeding and strain selection

Another approach to increase the yield of laccase using classical selective breeding and strain selection was also reported recently. Eichlerova and Homolka (1999) prepared new high laccase producing dikaryotic strains of *P. ostreatus* by crossing of monokaryons derived from randomly chosen basidiospores (spores harvested from fruiting body of mushrooms) obtained from a parental strain *P. ostreatus* Florida f6. The authors prepared and characterised 30 isolates of *P. ostreatus* monokaryons, some of which had improved laccase, MnP and Mn-independent peroxidase producing abilities, and subsequently prepared a number of stable dikaryons after compatible crossing of monokaryons. In some of the dikaryons, the enzyme producing ability was improved further. The authors suggested the possibility of preparing better laccase producing strains from other dikaryotic strains and other basidiomycetes using this approach. Herpoel *et al.* (2000) carried out a similar experiment with 8 different *P. cinnabarinus* strains and isolated 48 monokaryotic strains. The authors could find one monokaryon strain, ss3, with much higher laccase producing ability than its parental strain. The same *P. cinnabarinus* monokaryon strain was used recently for high production of laccase (266.6 U mL^{-1}) in shaken cultures using ethanol as an inducer (Lomascolo *et al.*, 2003). A similar classical genetic method was also used to isolate a hyper-laccase producing monokaryon strain of *P. sanguineus* screened from a number of *Pycnoporus* species collected around the world (Lomascolo *et al.*, 2002).

3.5.2.4. Optimisation of growth conditions

Several groups of researchers attempted to optimise the production of laccase by either conventional or statistical methods. Medeiros *et al.* (1999) optimised laccase production by *P. ostreatus* DM-1513 using a full 2^5 factorial design followed by the path of steepest ascent experiments. The authors found that low pH and high yeast extract concentration without the use of an inducer (unspecified compound) and buffer system had positive effects on the laccase production. Vasconcelos *et al.* (2000) utilised factorial design and surface response method to determine optimum concentrations of veratryl alcohol and yeast extract, optimum time of cultivation and agitation speed for the production of laccase by *Botryosphaeria* sp. MAMB-5. The authors found that 30.4 mM veratryl alcohol, 4.5 days at 28°C with an agitation speed of 180 rpm were the optimal conditions to maximise the production of one of the laccase isoenzymes produced by this fungus. Galhaup *et al.* (2002) studied the effects of various culture conditions on the production of laccase by *T. pubescens* and found the following optimised growth conditions: 40 g L^{-1} glucose, 10 g L^{-1} peptone from meat, 2.0 mM Cu(II) and 1 g L^{-1} $MgSO_4 \cdot 7H_2O$. The authors also found that a fed batch operation of fermentation increased the laccase production by *T. pubescens* by two fold and obtained the remarkably high laccase activity of 740 U mL^{-1}.

A group of researchers recently investigated the selection of solid substrates for fungal immobilisation, inducers and bioreactor configurations for the laccase production by *T. versicolor* CBS100.29. Couto *et al.* (2002d; 2003) evaluated four natural solids, including wheat straw, barley straw, wood shavings and barley bran, as well as two inert supports, polyurethane foam and nylon sponge, for solid-state fermentation of *T. versicolor* and found that laccase activity was highest in the solid-state culture containing barley bran. The authors also noted that xylidine was a better inducer than veratryl alcohol for laccase production. Three bioreactor configurations were tested for the solid-state fermentation of *T. versicolor* to produce laccase, including immersion, expanded-bed and tray bioreactors (Couto *et al.*, 2003). The authors found the best results in the tray bioreactor filled with barley bran as a support.

3.6. Concluding remarks

Numerous reports have been published on the production of extracellular fungal peroxidases, especially LiP and MnP, and laccases for their industrial and environmental applications during the last several years. One apparent trend in the current literature is the discovery of new isolates of fungi that exhibit improved production of these enzymes. Although this trend may still continue for a while, the overall impact of such studies on the development of enzyme production for industrial and environmental scale may be relatively small. Reducing the cost for enzyme production is still needed in order to develop enzymatic treatment processes, which are more competitive with conventional and other novel treatment technologies. This may be achieved by means of the process optimisation using statistical experimental designs, which is also called process engineering, and the use of cheaper growth substrates such as agricultural and food wastes (Pandey *et al.*, 1999).

Although not dealt with in this review, overexpression of recombinant enzymes in heterologous systems, such as yeast and bacteria, has been actively studied to enhance the production of fungal peroxidases and laccases, as well as to improve the catalytic activity and stability of these enzymes, during the last several years. Whereas these attempts have yielded limited success on improving enzyme production so far (Conesa *et al.*, 2002), the recent advances in biotechnology may overcome the difficulties and may lead a major breakthrough for the production of industrially and environmentally important enzymes, including fungal peroxidases and laccases.

References

Aitken, M. D. (1993). Waste treatment applications of enzymes: opportunities and obstacles. *The Chemical Engineering Journal* **52**: B49-B58.

Aitken, M. D., Venkatadri, R. and Irvine, R. L. (1989). Oxidation of phenolic pollutant by a lignin degrading enzyme from the white-rot fungus *Phanerochaete chrysosporium*. *Water Research* **23**(4): 443-450.

Akimoto, K., Sinmen, Y., Sumida, M., Asami, S., Amachi, T., Yoshizumi, H., Saeki, Y., Shimizu, S. and Yamada, H. (1990). Luminol chemiluminescence reaction catalyzed by a microbial peroxidase. *Analytical Biochemistry* **189**: 182-185.

Al-Kassim, L., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1993). Aromatic removal from water by *Arthromyces ramosus* peroxidase. in *Plant Peroxidases: Biochemistry and Physiology*. Welinder, K. G., Rasmussen, S. K., Penel, C. and Greppin, H., Editors, University of Geneva, Geneva: 197-200.

Al-Kassim, L., Taylor, K. E., Nicell, J. A., Bewtra, J. K. and Biswas, N. (1994). Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxide from *Coprinus macrorhizus* in batch reactors. *Journal of Chemical Technology and Biotechnology* **61**: 179-182.

Annadurai, G., Babu, S. R., Nagarajan, G. and Ragu, K. (2000). Use of Box-Behnken design of experiments in the production of manganese peroxidase by *Phanerochaete chrysosporium* (MTCC 767) and decolorization of crystal violet. *Bioprocess Engineering* **23**: 715-719.

Archibald, F., Paice, M. G. and Jurasek, L. (1990). Decolorization of kraft bleachery effluent chromophores by *Coriolus (Trametes) versicolor*. *Enzyme and Microbial*

Technology **12**: 846-853.

Arora, D. S. and Gill, P. K. (2001). Effects of various media and supplements on laccase production by some white rot fungi. *Bioresource Technology* **77**: 89-91.

Arora, D. S. and Rampal, P. (2002). Laccase production by some *Phlebia* species. *Journal of Basic Microbiology* **42**(5): 295-301.

Bajpai, P. (1999). Application of enzymes in the pulp and paper industry. *Biotechnology Progress* **15**(2): 147-157.

Baunsgaard, L., Dalboge, H., Houen, G., Rasmussen, E. M. and Welinder, K. G. (1993). Amino acid sequence of *Coprinus macrorhizus* peroxidase and cDNA sequence encoding *Coprinus cinereus* peroxidase. A new family of fungal peroxidases. *European Journal of Biochemistry* **213**: 605-611.

Bollag, J.-M. and Leonowicz, A. (1984). Comparative studies of extracellular fungal laccases. *Applied and Environmental Microbiology* **48**(4): 849-854.

Bollag, J.-M., Shuttleworth, K. L. and Anderson, D. H. (1988). Laccase-mediated detoxificaion of phenolic compounds. *Applied and Environmental Microbiology* **54**(12): 3086-3091.

Bonnarme, P. and Jeffries, T. W. (1990). Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white rot fungi. *Applied and Environmental Microbiology* **56**(1): 210-217.

Bourbonnais, R., Leech, D. and Paice, M. G. (1998). Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. *Biochimica et Biophysica Acta* **1379**: 381-390.

Bourbonnais, R. and Paice, M. G. (1990). Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Letters* **267**(1): 99-102.

Burke, R. M. and Cairney, J. W. G. (2002). Laccases and other polyphenol oxidases in ecto- and ericoid mycorrhizal fungi. *Mycorrhiza* **12**(3): 105-116.

Buswell, J. A., Mollet, B. and Odier, E. (1984). Ligninolytic enzyme production by *Phanerochaete chrysosporium* under conditions of nitrogen sufficiency. *FEMS Microbiology Letters* **25**: 295-299.

Cabaleiro, D. R., Rodriguez-Couto, S., Sanroman, A. and Longo, M. A. (2002). Comparison between the protease production ability of ligninolytic fungi cultivated in solid state media. *Process Biochemistry* **37**: 1017-1023.

Cambria, M. T., Cambria, A., Ragusa, S. and Rizzarelli, E. (2000). Production, purification, and properties of an extracellular laccase from *Rigidoporus lignosus*. *Protein Expression and Purification* **18**: 141-147.

Caramelo, L., Martinez, M. J. and Martinez, A. T. (1999). A search for ligninolytic peroxidases in the fungus *Pleurotus eryngii* involving α-keto-γ-thiomethylbutyric acid and lignin model dimers. *Applied and Environmental Microbiology* **65**(3): 916-922.

Casella, L. and Marchesini, A. (1994). The chloroperoxidase-catalyzed oxidation of phenols. Mechanism, selectivity and characterization of enzyme-substrate complexes. *Biochemistry* **33**(21): 6377-6386.

Cassland, P. and Jonsson, L. J. (1999). Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature. *Applied Microbiology and Biotechnology* **52**: 393-400.

Castro-Sowinski, S., Martinez-Drets, G. and Okon, Y. (2002). Laccase activity in melanin-producing strains of *Sinorhizobium meliloti*. *FEMS Microbiology*

Letters **209**: 119-125.

Collins, P. J. and Dobson, A. D. W. (1997). Regulation of laccase gene transcription in *Trametes versicolor*. *Applied and Environmental Microbiology* **63**(9): 3444-3450.

Collins, P. J., Kotterman, M. J. J., Field, J. A. and Dobson, A. D. W. (1996). Oxidation of anthracene and benzo[*a*]pyrene by laccases from *Trametes versicolor*. *Applied and Environmental Microbiology* **62**(12): 4563-4567.

Conesa, A., Punt, P. J. and van den Hondel, C. A. M. J. J. (2002). Fungal peroxidases: molecular aspects and applications. *Journal of Biotechnology* **93**: 143-158.

Couto, S. R., Barreiro, M., Rivela, I., Longo, M. A. and Sanroman, A. (2002a). Performance of a solid-state immersion bioreactor for ligninolytic enzyme production: evaluation of different operational variables. *Process Biochemisty* **38**: 219-227.

Couto, S. R., Dominguez, A. and Sanroman, A. (2002b). Production of manganese-dependent peroxidase in a new solid-state bioreactor by *Phanerochaete chrysosporium* grown on wood shavings. Application to the decolorization of synthetic dyes. *Folia Microbiologica* **47**: 417-421.

Couto, S. R., Feijoo, G., Moreira, M. T. and Lema, J. M. (2002c). Evaluation of the environmental conditions for the continuous production of lignin peroxidase by *Phanerochaete chrysosporium* in fixed-bed bioreactors. *Biotechnology Letters* **24**: 791-794.

Couto, S. R., Gundin, M., Lorenzo, M. and Sanroman, M. N. (2002d). Screening of supports and inducers for laccase production by *Trametes versicolor* in semi-solid-state conditions. *Process Biochemistry* **38**: 249-255.

Couto, S. R., Moldes, D., Liebanas, A. and Sanroman, A. (2003). Investigation of several bioreactor configurations for laccase production by *Trametes versicolor* operating in solid-state conditions. *Biochemical Engineering Journal* **15**: 21.

Couto, S. R., Ratto, M., Dominguez, A. and Sanroman, A. (2001). Strategies for improving ligninolytic enzyme activities in semi-solid-state bioreactors. *Process Biochemistry* **36**: 995-999.

Couto, S. R., Rivela, I., Munoz, M. R. and Sanroman, A. (2000). Ligninolytic enzyme production and the ability of decolourisation of Poly R-478 in packed-bed bioreactors by *Phanerochaete chrysosporium*. *Bioprocess Engineering* **23**: 287-293.

Dahiya, J. S., Singh, D. and Nigam, P. (1998). Characterisation of laccase produced by *Coniothyrium minitans*. *Journal of Basic Microbiology* **38**(5-6): 349-359.

Das, N., Chakraborty, T. K. and Mukherjee, M. (1999). Role of potato extract in extracellular laccase production of *Pleurotus florida*. *Journal of Basic Microbiology* **39**(5-6): 299-303.

De Jong, E., De Vries, F. P., Field, J. A., Van Der Zwan, R. P. and De Bont, J. A. M. (1992). Isolation and screening of basidiomycetes with high peroxidative activity. *Mycological Research* **96**(12): 1098-1104.

De Souza, C. G. M., Zilly, A. and Peralta, R. M. (2002). Production of laccase as the sole phenoloxidase by a Brazilian strain of *Pleurotus pulmonarius* in solid state fermentation. *Journal of Basic Microbiology* **42**(2): 83-90.

De Vries, O. M. H., Kooistra, W. H. C. F. and Wessels, J. G. H. (1986). Formation of an extracellular laccase by a *Schizophyllum commune* dikaryon. *Journal of General Microbiology* **132**: 2817-2826.

Dekker, R. F. H. and Barbosa, A. M. (2001). The effects of aeration and veratryl alcohol on the production of two laccases by the ascomycete *Botryosphaeria* sp. *Enzyme and Microbial Technology* **28**: 81-88.

Dekker, R. F. H., Vasconcelos, A.-F. D., Barbosa, A. M., Giese, E. C. and Paccola-Meirelles, L. (2001). A new role for veratryl alcohol: regulation of synthesis of lignocellulose-degrading enzymes in the ligninolytic ascomyceteous fungus, *Botryosphaeria* sp.; influence of carbon source. *Biotechnology Letters* **23**: 1987-1993.

Dezotti, M., Innocentini-Mei, L. H. and Duran, N. (1995). Silica immobilized enzyme catalyzed removal of chlorolignins from eucalyptus kraft effluent. *Journal of Biotechnology* **43**: 161-167.

Dhawan, S. and Kuhad, R. C. (2002). Effect of amino acids and vitamins on laccase production by the bird's nest fungus *Cyathus bulleri*. *Bioresource Technology* **84**: 35-38.

Dhawan, S., Lal, R. and Kuhad, R. C. (2003). Ethidium bromide stimulated hyper laccase production from bird's nest fungus *Cyathus bulleri*. *Letters in Applied Microbiology* **36**: 64-67.

Dodson, P. J., Evans, C. S., Harvey, P. J. and Palmer, J. M. (1987). Production and properties of an extracellular peroxidase from *Coriolus versicolor* which catalyses C_{α}-C_{β} cleavage in a lignin model compound. *FEMS Microbiology Letters* **42**: 17-22.

Dominguez, A., Couto, S. R. and Sanroman, A. (2001a). Amelioration of ligninolytic enzyme production by *Phanerochaete chrysosporium* in air lift bioreactor. *Biotechnology Letters* **23**: 451-455.

Dominguez, A., Rivela, I., Couto, S. R. and Sanroman, M. A. (2001b). Design of a new rotating drum bioreactor for ligninolytic enzyme production by *Phanerochaete chrysosporium* grown on an inert support. *Process Biochemistry* **37**: 549-554.

D'Souza, T. M., Merritt, C. S. and Reddy, C. A. (1999). Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*. *Applied and Environmental Microbiology* **65**(12): 5307-5313.

Dunford, H. B. (1999). *Heme Peroxidases*. New York, NY, John Wiley & Sons, Inc.

Duran, N. and Esposito, E. (2000). Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Applied Catalysis B: Environmental* **28**: 83-99.

Duran, N., Ferrer, I. and Rodriguez, J. (1987a). Ligniases from *Chrysonilia sitophila* (TFB-27441 strain). *Applied Biochemistry and Biotechnology* **16**: 157-167.

Duran, N., Rodriguez, J., Ferraz, A. and Campos, V. (1987b). *Chrysonila sitophila* (TFB-27441): A hyperlignolytic strain. *Biotechnology Letters* **9**(5): 357-360.

Eaton, D., Chang, H.-m. and Kirk, T. K. (1980). Fungal decolorization of kraft bleach plant effluents. *Tappi* **63**(10): 103-106.

Eggert, C., Temp, U., Dean, J. F. D. and Eriksson, K.-E. L. (1996a). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Letters* **391**: 144-148.

Eggert, C., Temp, U. and Eriksson, K.-E. L. (1996b). The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: Purification and characterization of the laccase. *Applied and Environmental Microbiology* **62**(4): 1151-1158.

Eggert, C., Temp, U. and Eriksson, K.-E. L. (1997). Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*. *FEBS Letters*

407: 89-92.

Eichlerova, I. and Homolka, L. (1999). Preparation and crossing of basidiospore-derived monokaryons - a useful tool for obtaining laccase and other ligninolytic enzyme higher-producing dikaryotic strains of *Pleurotus ostreatus*. *Antonie van Leeuwenhoek* **75**: 321-327.

Eichlerova, I., Homolka, L., Nerud, F., Zadrazil, F., Baldrian, P. and Gabriel, J. (2000). Screening of *Pleurotus ostreatus* isolates for their ligninolytic properties during cultivation on natural substrates. *Biodegradation* **11**(5): 279-287.

Fahraeus, G. and Reinhammar, B. (1967). Large scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A. *Acta Chemica Scandinavica* **21**: 2367-2378.

Faison, B. D. and Kirk, T. K. (1985). Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **49**(2): 299-304.

Fenice, M., Sermanni, G. G., Federici, F. and D'Annibale, A. (2003). Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. *Journal of Biotechnology* **100**: 77-85.

Ferreira, V. S., Magalhaes, D. B., Kling, S. H., G., D. S. J. J. and Bon, E. P. S. (2000). *N*-Demethylation of methylene blue by lignin peroxidase from *Phanerochaete chrysosporium* - Stoichiometric relation for H_2O_2 consumption. *Applied Biochemistry and Biotechnology* **84-86**: 255-265.

Galhaup, C. and Haltrich, D. (2001). Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Applied Microbiology and Biotechnology* **56**: 225-232.

Galhaup, C., Wagner, H., Hinterstoisser, B. and Haltrich, D. (2002). Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. *Enzyme and Microbial Technology* **30**: 529-536.

Galliano, H., Gas, G., Seris, J. L. and Boudet, A. M. (1991). Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. *Enzyme and Microbial Technology* **13**: 478-482.

Giardina, P., Palmieri, G., Fontanella, B., Rivieccio, V. and Sannia, G. (2000). Manganese peroxidase isoenzymes produced by *Pleurotus ostreatus* grown on wood sawdust. *Archives of Biochemistry and Biophysics* **376**(1): 171-179.

Gill, P. K. and Arora, D. S. (2003). Effect of culture conditions on manganese peroxidase production and activity by some white rot fungi. *Journal of Industrial Microbiology and Biotechnology* **30**: 28-33.

Glenn, J. K., Aran, L. A. and Gold, M. H. (1986). Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics* **251**(2): 688-696.

Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M. and Gold, M. H. (1983). An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. *Biochemical and Biophysical Research Communications* **114**(3): 1077-1083.

Gold, M. H., Kuwahara, M., Chiu, A. A. and Glenn, J. K. (1984). Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white rot basidiomycete *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics* **234**(2): 353-362.

Gomez-Toribio, V., Martinez, A. T., Martinez, M. J. and Guillen, F. (2001). Oxidation of hydroquinones by the versatile ligninolytic peroxidase from *Pleurotus eryngii.* H_2O_2 generation and the influence of Mn^{2+}. *European Journal of Biochemistry* **268**: 4787-4793.

Govender, S., Jacobs, E. P., Leukes, W. D. and Pillay, V. L. (2003). A scalable membrane gradostat reactor for enzyme production using *Phanerochaete chrysosporium. Biotechnology Letters* **25**: 127-131.

Grgic, I. and Perdih, A. (2003). Stimulation of ligninolytic enzyme production in *Phanerochaete chrysosporium* by polyoxyalkanes. *Journal of Applied Microbiology* **94**: 360-368.

Guillen, F., Gomez-Toribio, V., Martinez, M. J. and Martinez, A. T. (2000). Production of hydroxyl radical by the synergistic action of fungal laccase and aryl alcohol oxidase. *Archives of Biochemistry and Biophysics* **383**(1): 142-147.

Guillen, F., Martinez, M. J., Munoz, C. and Martinez, A. T. (1997). Quinone redox cycling in the ligninolytic fungus *Pleurotus eryngii* leading to extracellular production of superoxide anion radical. *Archives of Biochemistry and Biophysics* **339**(1): 190-199.

Ha, H.-C., Honda, Y., Watanabe, T. and Kuwahara, M. (2001). Production of manganese peroxidase by pellet culture of the lignin-degrading basidiomycete, *Pleurotus ostreatus. Applied Microbiology and Biotechnology* **55**: 704-711.

Haemmerli, S. D., Leisola, M. S. A. and Fiechter, A. (1986a). Polymerisation of lignins by ligninases from *Phanerochaete chrysosporium. FEMS Microbiology Letters* **35**: 33-36.

Haemmerli, S. D., Leisola, M. S. A., Sanglard, D. and Fiechter, A. (1986b). Oxidation of benzo(*a*)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. *The Journal of Biological Chemistry* **261**(15): 6900-6903.

Hamman, O. B., de la Rubia, T. and Martinez, J. (1999). The effect of manganese on the production of *Phanerochaete flavido-alba* ligninolytic peroxidases in nitrogen limited cultures. *FEMS Microbiology Letters* **177**: 137-142.

Hammel, K. E., Kalyanaraman, B. and Kirk, T. K. (1986). Oxidation of polycyclic aromatic hydrocarbons and dibenzo[*p*]-dioxins by *Phanerochaete chrysosporium* ligniase. *The Journal of Biological Chemistry* **261**(36): 16948-16952.

Hammel, K. E. and Tardone, P. J. (1988). The oxidative 4-dechlorination of polychlorinated phenols is catalyzed by extracellular fungal lignin peroxidases. *Biochemistry* **27**(17): 6563-6568.

Harvey, P. J., Palmer, J. M., Schoemaker, H. E., Dekker, H. L. and Wever, R. (1989). Pre-steady-state kinetic study on the formation of compound I and II of ligninase. *Biochimica et Biophysica Acta* **994**: 59-63.

Harvey, P. J., Schoemaker, H. E. and Palmer, J. M. (1986). Veratryl alcohol as a mediator and the role of radical cations in lignin biodegradation by *Phanerochaete chrysosporium*. *FEBS Letters* **195**(1-2): 242-246.

Hatvani, N. and Mecs, I. (2001). Production of laccase and manganese peroxidase by *Lentinus edodes* on malt-containing by-product of the brewing process. *Process Biochemistry* **37**: 491-496.

Hatvani, N. and Mecs, I. (2002). Effect of the nutrient composition on dye decolorisation and extracellular enzyme production by *Lentinus edodes* on solid medium. *Enzyme and Microbial Technology* **30**: 381-386.

Herpoel, I., Asther, M. and Sigoillot, J.-C. (1999). Design and scale up of a process for manganese peroxidase production using the hypersecretory strain *Phanerochaete chrysosporium* I-1512. *Biotechnology and Bioengineering* **65**(4): 468-473.

Herpoel, I., Moukha, S., Lesage-Meessen, L., Sigoillot, J.-C. and Asther, M. (2000). Selection of *Pycnoporus cinnabarinus* strains for laccase production. *FEMS Microbiology Letters* **183**: 301-306.

Hess, J., Leitner, C., Galhaup, C., Kulbe, K. D., Hinterstoisser, B., Steinwender, M. and Haltrich, D. (2002). Enhanced formation of extracellular laccase activity by the white-rot fungus *Trametes multicolor*. *Applied Biochemistry and Biotechnology* **98-100**: 229-241.

Higuchi, T. (1990). Lignin biochemistry: Biosynthesis and biodegradation. *Wood Science and Technology* **24**: 23-63.

Hirano, T., Honda, Y., Watanabe, T. and Kuwahara, M. (2000). Degradation of bisphenol A by the lignin-degrading enzyme, manganese peroxidase, produced by the white-rot basidiomycete, *Pleurotus ostreatus*. *Bioscience, Biotechnology, and Biochemistry* **64**(9): 1958-1962.

Hofrichter, M., Vares, T., Kalsi, M., Galkin, S., Scheibner, K., Fritsche, W. and Hatakka, A. (1999). Production of manganese peroxidase and organic acids and mineralization of ^{14}C-labelled lignin (^{14}C-DHP) during solid-state fermentation of wheat straw with the white rot fungus *Nematoloma frowardii*. *Applied and Environmental Microbiology* **65**(5): 1864-1870.

Hong, F., Meinander, N. Q. and Jonsson, L. J. (2002). Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnology and Bioengineering* **79**: 438-449.

Huynh, V.-B. and Crawford, R. L. (1985). Novel extracellular enzymes (ligninases) of

Phanerochaete chrysosporium. *FEMS Microbiology Letters* **28**: 119-123.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (2001). Enzyme-catalyzed removal of phenol from refinery wastewater: Feasibility studies. *Water Environment Research* **73**(2): 165-172.

Ikehata, K. and Buchanan, I. D. (2002). Screening of *Coprinus* species for the production of extracellular peroxidase and evaluation of the enzyme for the treatment of aqueous phenol. *Environmental Technology* **23**(12): 1355-1368.

Ikehata, K., Buchanan, I. D. and Smith, D. W. (2003a). Treatment of oil refinery wastewater using crude *Coprinus cinereus* peroxidase and hydrogen peroxide. *Journal of Environmental Engineering and Science* (Accepted).

Ikehata, K., Buchanan, I. D. and Smith, D. W. (2003b). Enzymatic treatment of phenol with crude fungal peroxidase from *Coprinus* species. *Abstracts of Papers: 225th ACS National Meeting, Biochemical Technology Division Symposia*, New Orleans, LA, American Chemical Society, Washington, DC: BIOT18.

Jang, M. Y., Ryu, W. R. and Cho, M. H. (2002). Laccase production from repeated batch cultures using free mycelia of *Trametes* sp. *Enzyme and Microbial Technology* **30**: 741-746.

Jeffries, T. W., Choi, S. and Kirk, T. K. (1981). Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **42**(2): 290-296.

Johannes, C. and Majcherczyk, A. (2000). Natural mediators in the oxidation of polycyclinc aromatic hydrocarbons by laccase mediator systems. *Applied and Environmental Microbiology* **66**(2): 524-528.

Jung, H., Xu, F. and Li, K. (2002). Purification and characterization of laccase from wood-degrading fungus *Trichophyton rubrum* LKY-7. *Enzyme and Microbial Technology* **30**: 161-168.

Jurasek, L. and Paice, M. (1986). Pulp, paper, and biotechnology. *Chemtech* **16**(6): 360-365.

Kaal, E. E. J., De Jong, E. and Field, J. A. (1993). Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. *Applied and Environmental Microbiology* **59**(12): 4031-4036.

Kahraman, S. S. and Gurdal, I. H. (2002). Effect of synthetic and natural culture media on laccase production by white rot fungi. *Bioresource Technology* **82**: 215-217.

Kaluskar, V. M., Kapadnis, B. P., Jaspers, C. and Penninckx, M. J. (1999). Production of laccase by immobilized cells of *Agaricus* sp. Induction effect of xylan and lignin derivatives. *Applied Biochemistry and Biotechnology* **76**: 161-170.

Karam, J. and Nicell, J. A. (1997). Potential application of enzymes in waste treatment. *Journal of Chemical Technology and Biotechnology* **69**: 141-153.

Kauffmann, C., Petersen, B. R. and Bjerrum, M. J. (1999). Enzymatic removal of phenols from aqueous solutions by *Coprinus cinereus* peroxidase and hydrogen peroxide. *Journal of Biotechnology* **73**: 71-74.

Kawai, S., Umezawa, T. and Higuchi, T. (1988). Degradation mechanisms of phenolic β-1 lignin substructure model compounds by laccase of *Coriolus versicolor*. *Archives of Biochemistry and Biophysics* **262**(1): 99-110.

Kim, B. B., Pisarev, V. V. and Egorov, A. M. (1991). A comparative study of peroxidases from horse radish and *Arthromyces ramosus* as labels in luminol-mediated chemiluminescent assays. *Analytical Biochemistry* **199**: 1-6.

Kimura, Y., Asada, Y. and Kuwahara, M. (1990). Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. *Applied Microbiology and Biotechnology* **32**: 436-442.

Kirk, T. K., Harkin, J. M. and Cowling, E. B. (1968). Degradation of the lignin model compound syringylglycol-β-guaiacyl ether by *Polyporous versicolor* and *Sterium frustulatum*. *Biochimica et Biophysica Acta* **165**: 145-163.

Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F. and Zeikus, J. G. (1978). Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. *Archives of Microbiology* **117**: 277-285.

Kirk, T. K., Tien, M., Kersten, P. J., Mozuch, M. D. and Kalyanaraman, B. (1986). Ligninase of *Phanerochaete chrysosporium* Mechanism of its degradation of the non-phenolic arylglycerol *β*-aryl ether substrate of lignin. *Biochemical Journal* **236**: 279-287.

Kjalke, M., Andersen, M. B., Schneider, P., Christensen, B., Schulein, M. and Welinder, K. G. (1992). Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. *Biochimica et Biophysica Acta* **1120**: 248-256.

Klonowska, A., Le Petit, J. and Tron, T. (2001). Enhancement of minor laccases production in the basidiomycete *Marasmius quercophilus* C30. *FEMS Microbiology Letters* **200**: 25-30.

Kluczek-Turpeinen, B., Tuomela, M., Hatakka, A. and Hofrichter, M. (2003). Lignin degradation in a compost environment by the deuteromycete *Paecilomyces inflatus*. *Applied Microbiology and Biotechnology* **61**: 374-379.

Koroleva, O. V., Gavrilova, V. P., Stepanova, E. V., Lebedeva, V. I., Sverdlova, N. I., Landesman, E. O., Yavmetdinov, I. S. and Yaropolov, A. I. (2002a). Production of lignin modifying enzymes by co-cultivated white-Rot Fungi *Cerrena maxima* and *Coriolus hirsutus* and characterization of laccase from *Cerrena maxima*. *Enzyme and Microbial Technology* **30**: 573-580.

Koroleva, O. V., Stepanova, E. V., Gavrilova, V. P., Yakovleva, N. S., Landesman, E. O., Yavmetdinov, I. S. and Yaropolov, A. I. (2002b). Laccase and Mn-peroxidase production by *Coriolus hirsutus* strain 075 in a jar fermentor. *Journal of Bioscience and Bioengineering* **93**(5): 449-455.

Koroljova-Skorobogat'ko, O. V., Stepanova, E. V., Gavrilova, V. P., Morozova, O. V., Lubimova, N. V., Dzchafarova, A. N., Jaropolov, A. I. and Makower, A. (1998). Purification and characterization of the constitutive form of laccase from the basidiomycete *Coriolus hirsutus* and effect of inducers on laccase synthesis. *Biotechnology and Applied Biochemistry* **28**: 47-54.

Kunishima, N., Fukuyama, K., Matsubara, H., Hatanaka, H., Shibano, Y. and Amachi, T. (1994). Crystal structure of the fungal peroxidase from *Arthromyces ramosus* at 1.9 A resolution. *Journal of Molecular Biology* **235**(1): 331-344.

Kuwahara, M., Glenn, J. K., Morgan, M. A. and Gold, M. H. (1984). Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. *FEBS Letters* **169**(2): 247-250.

Kwon, S.-I. and Anderson, A. J. (2001). Laccase isozymes: production by an opportunistic pathogen, a *Fusarium proliferatum* isolate from wheat. *Physiological and Molecular Plant Pathology* **59**: 235-242.

Lee, I.-Y., Jung, K.-H., Lee, C.-H. and Park, Y.-H. (1999). Enhanced production of laccase in *Trametes versicolor* by the addition of ethanol. *Biotechnology Letters* **21**: 965-968.

Leisola, M. S. A. and Fiechter, A. (1985). Ligninase production in agitated conditions by *Phanerochaete chrysosporium*. *FEMS Microbiology Letters* **29**: 33-36.

Leisola, M. S. A., Schmidt, B., Thanei-Wyss, U. and Fiechter, A. (1985). Aromatic ring cleavage of veratryl alcohol by *Phanerochaete chrysosporium*. *FEBS Letters* **189**(2): 267-270.

Leonowicz, A., Edgehill, R. U. and Bollag, J.-M. (1984). The effect of pH on the transformation of syringic and vanillic acids by the laccases of *Rhizoctonia praticola* and *Trametes versicolor*. *Archives of Microbiology* **137**: 89-96.

Leonowicz, A., Szklarz, G. and Wojtas-Wasilewska, M. (1985). The effect of fungal laccase on fractionated lignosulphonates (Peritan Na). *Phytochemistry* **24**(3): 393-396.

Levin, L. and Forchiassin, F. (2001). Ligninolytic enzymes of the white rot basidiomycete *Trametes trogii*. *Acta Biotechnologica* **21**(2): 179-186.

Levin, L., Forchiassin, F. and Ramos, A. M. (2002). Copper induction of lignin-modifying enzymes in the white-rot fungus *Trametes trogii*. *Mycologia* **94**(3): 377-383.

Littlechild, J. (1999). Haloperoxidases and their role in biotransformation reactions. *Current Opinion in Chemical Biology* **3**: 28-34.

Lo, S. C., Ho, Y. S. and Buswell, J. A. (2001). Effect of phenolic monomers on the production of laccases by the edible mushroom *Pleurotus sajor-caju*, and partial characterization of a major laccase component. *Mycologia* **93**(3): 413-421.

Lomascolo, A., Cayol, J. L., Roche, M., Guo, L., Robert, J. L., Record, E., Lesage-Meessen, L., Ollivier, B., Sigoillot, J. C. and Asther, M. (2002). Molecular clustering of *Pycnoporus* strains from various geographic origins and isolation of monokaryotic strains for laccase hyperproduction. *Mycological Research* **106**(10): 1193-1203.

Lomascolo, A., Record, E., Herpoel-Gimbert, I., Delattre, M., Robert, J. L., Georis, J., Dauvrin, T., Sigoillot, J. C. and Asther, M. (2003). Overproduction of laccase by a monokaryotic strain of *Pycnoporus cinnabarinus* using ethanol as inducer. *Journal of Applied Microbiology* **94**: 618-624.

Lorenzo, M., Moldes, D., Couto, S. R. and Sanroman, A. (2002). Improving laccase production by employing different lignocellulosic wastes in submerged cultures of *Trametes versicolor*. *Bioresource Technology* **82**: 109-113.

Maceiras, R., Rodriguez-Couto, S. and Sanroman, A. (2001). Influence of several activities on the extracellular laccase activity and *in vivo* decolourization of Poly R-478 by semi-solid-state cultures of *Trametes versicolor*. *Acta Biotechnologica* **21**(3): 255-264.

Manimekalai, R. and Swaminathan, T. (1999). Optimisation of lignin peroxidase production from *Phanerochaete chrysosporium* using response surface methodology. *Bioprocess Engineering* **21**: 465-468.

Martinez, A. T. (2002). Molecular biology and structure-function of lignin-degrading heme peroxidases. *Enzyme and Microbial Technology* **30**: 425-444.

Marzullo, L., Cannio, R., Giardina, P., Santini, M. T. and Sannia, G. (1995). Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and prevents polymerization of laccase-oxidized substrates. *The Journal of Biological Chemistry* **270**(8): 3823-3827.

Medeiros, M. B., Bento, A. V., Nunes, A. L. L. and Oliveira, S. C. (1999). Optimization of some variables that affect the synthesis of laccase by *Pleurotus ostreatus*.

Bioprocess Engineering **21**: 483-487.

Mester, T. and Field, J. A. (1997). Optimization of manganese peroxidase production by the white rot fungus *Bjerkandera* sp. strain BOS55. *FEMS Microbiology Letters* **155**: 161-168.

Mester, T. and Field, J. A. (1998). Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. *The Journal of Biological Chemistry* **273**(25): 15412-15417.

Mester, T., Pena, M. and Field, J. A. (1996). Nutrient regulation of extracellular peroxidases in the white rot fungus, *Bjerkandera* sp. strain BOS55. *Applied Microbiology and Biotechnology* **44**: 778-784.

Mileski, G. J., Bumpus, J. A., Jurek, M. A. and Aust, S. D. (1988). Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **54**(12): 2885-2889.

Moldes, D., Couto, S. R., Cameselle, C. and Sanroman, M. A. (2003). Study of the degradation of dyes by MnP of *Phanerochaete chrysosporium* produced in a fixed-bed bioreactor. *Chemosphere* **51**: 295-303.

Morais, H., Ramos, A. C., Cserhati, T., Forgacs, E., Darwish, Y. and Illes, Z. (2001a). Effect of the composition of culture media on the laccase production of *Lentinus edodes* strains. *Acta Biotechnologica* **21**(4): 307-320.

Morais, H., Ramos, C., Forgacs, E., Cserhati, T. and Oliviera, J. (2001b). Lignin-modifying enzymes of *Pleurotus ostreatus* grown on agro-residues. *Acta Alimentaria* **30**(4): 363-372.

Moreira, M. T., Feijoo, G. and Lema, J. M. (2000a). Manganese peroxidase production by

Bjerkandera sp. BOS55. 1. Regulation of enzymatic production. *Bioprocess Engineering* **23**: 657-661.

Moreira, M. T., Torrado, A., Feijoo, G. and Lema, J. M. (2000b). Manganese peroxidase production by *Bjerkandera* sp. BOS55. 2. Operation in stirred tank reactors. *Bioprocess Engineering* **23**: 663-667.

Morita, Y., Yamashita, H., Mikami, B., Iwamoto, H., Aibara, S., Terada, M. and Minami, J. (1988). Purification, crystallization, and characterization of peroxidase from *Coprinus cinereus*. *Journal of Biochemistry* **103**(4): 693-699.

Mougin, C., Kollmann, A. and Jolivalt, C. (2002). Enhanced production of laccase in the fungus *Trametes versicolor* by the addition of xenobiotics. *Biotechnology Letters* **24**: 139-142.

Munoz, C., Guillen, F., Martinez, A. T. and Martinez, M. J. (1997a). Laccase isoenzymes of *Pleurotus eryngii*: Characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. *Applied and Environmental Microbiology* **63**(6): 2166-2174.

Munoz, C., Guillen, F., Martinez, A. T. and Martinez, M. J. (1997b). Induction and characterization of laccase in the ligninolytic fungus *Pleurotus eryngii*. *Current Microbiology* **34**: 1-5.

Nakamura, Y., Sungusia, M. G., Sawada, T. and Kuwahara, M. (1999). Lignin-degrading enzyme production by *Bjerkandera adusta* immobilized on polyurethane foam. *Journal of Bioscience and Bioengineering* **88**(1): 41-47.

Nakayama, T. and Amachi, T. (1999). Fungal peroxidase: its structure, function, and application. *Journal of Molecular Catalysis B-Enzymatic* **6**: 185-198.

Neri, F., Indiani, C., Baldi, B., Vind, J., Welinder, K. G. and Smulevich, G. (1999). Role of the distal phenylalanine 54 on the structure, stablity, and ligand binding of *Coprinus cinereus* peroxidase. *Biochemistry* **38**(24): 7819-7827.

Nerud, F., Zouchova, Z. and Misurcova, Z. (1991). Ligninolytic properties of different white-rot fungi. *Biotechnology Letters* **13**(9): 657-660.

Nicell, J. A. (2001). Environmental applications of enzymes. *Interdisciplinary Environmental Review* **3**(1): 14-41.

Niku-Paavola, M.-L., Karhunen, E., Salola, P. and Raunio, V. (1988). Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochemical Journal* **254**: 877-884.

Novotny, C., Erbanova, P., Sasek, V., Kubatova, A., Cajthaml, T., Lang, E., Krahl, J. and Zadrazil, F. (1999). Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. *Biodegradation* **10**: 159-168.

Nuske, J., Scheibner, K., Dornberger, U., Ullrich, R. and Hofrichter, M. (2002). Large scale production of manganese-peroxidase using agaric white-rot fungi. *Enzyme and Microbial Technology* **30**: 556-561.

Nyanhongo, G. S., Gomes, J., Gubitz, G., Zvauya, R., Read, J. S. and Steiner, W. (2002a). Production of laccase by a newly isolated strain of *Trametes modesta*. *Bioresource Technology* **84**(3): 259-263.

Nyanhongo, G. S., Gomes, J., Gubitz, G. M., Zvauya, R., Read, J. and Steiner, W. (2002b). Decolorization of textile dyes by laccases from a newly isolated strain of *Trametes modesta*. *Water Research* **36**: 1449-1456.

Orth, A. B., Denny, M. and Tien, M. (1991). Overproduction of lignin-degrading enzymes by an isolate of *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **57**(9): 2591-2596.

Orth, A. B., Royse, D. J. and Tien, M. (1993). Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. *Applied and Environmental Microbiology* **59**(12): 4017-4023.

Orton, P. D. and Watling, R. (1979). Coprinaceae Part 1: Coprinus. in *British Fungus Flora Agarics and Boleti*. Henderson, D. M., Orton, P. D. and Watling, R., Editors. Royal Botanic Garden, Edinburgh, Her Majesty's Stationery Office: 7-125.

Pandey, A., Selvakumar, P., Soccol, C. R. and Nigam, P. (1999). Solid state fermentation for the production of industrial enzymes. *Current Science* **77**(1): 149-162.

Papinutti, V. L., Diorio, L. A. and Forchiassin, F. (2003). Production of laccase and manganese peroxidase by *Fomes sclerodermeus* grown on wheat bran. *Journal of Industrial Microbiology and Biotechnology* **30**: 157-160.

Paszczynski, A., Huynh, V.-B. and Crawford, R. (1985). Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium. FEMS Microbiology Letters* **29**: 37-41.

Paszczynski, A., Huynh, V.-B. and Crawford, R. (1986). Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium. Archives of Biochemistry and Biophysics* **244**(2): 750-765.

Pelaez, F., Martinez, M. J. and Martinez, A. T. (1995). Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation. *Mycological Research* **99**(1): 37-42.

Petersen, J. F. W., Kadziola, A. and Larsen, S. (1994). Three-dimensional structure of a recombinant peroxidase from *Coprinus cinereus* at 2.6 A resolution. *FEBS Letters* **339**: 291-296.

Pickard, M. A., Kadima, T. A. and Carmichael, R. D. (1991). Chloroperoxidase, a peroxidase with potential. *Journal of Industrial Microbiology* **7**: 235-242.

Pickard, M. A., Roman, R., Tinoco, R. and Vazquez-Duhalt, R. (1999a). Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. *Applied and Environmental Microbiology* **65**(9): 3805-3809.

Pickard, M. A., Vandertol, H., Roman, R. and Vazquez-Duhalt, R. (1999b). High production of ligninolytic enzymes from white rot fungi in cereal bran liquid medium. *Canadian Journal of Microbiology* **45**: 627-631.

Pointing, S. B., Jones, E. B. G. and Vrijmoed, L. L. P. (2000). Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. *Mycologia* **92**(1): 139-144.

Raghukumar, C. (2000). Fungi from marine habitats: an application in biodegradation. *Mycological Research* **104**(10): 1222-1226.

Raghukumar, C., D'Souza, T. M., Thorn, R. G. and Reddy, C. A. (1999). Lignin-modifiying enzymes of *Flavodon flavus*, a basidiomycete isolated from a coastal marine environment. *Applied and Environmental Microbiology* **65**(5): 2103-2111.

Ramachandra, M., Crawford, D. L. and Hertel, G. (1988). Characterization of an extracellular lignin peroxidase of lignocelluloytic actinomycete *Streptomyces viridosporus*. *Applied and Environmental Microbiology* **54**: 3057-3064.

Reddy, G. V., Babu, P. R., Komaraih, P., Roy, K. R. R. M. and Kothari, I. L. (2003). Utilization of banana waste for the production of lignolytic and cellulolytic enzymes by solid substrate fermentation using two *Pleurotus* species (*P.*

ostreatus and *P. sajor-caju*). *Process Biochemistry* **38**: 1457-1462.

Rivela, I., Couto, S. R. and Sanroman, A. (2000). Extracellular ligninolytic enzyme production by *Phanerochaete chrysosporium* in a new solid-state bioreactor. *Biotechnology Letters* **22**: 1443-1447.

Robles, A., Lucas, R., Cienfuegos, G. A. d. and Galvez, A. (2000). Phenol-oxidase (laccase) activity in strains of the hyphomycete *Chalara paradoxa* isolated from olive mill wastewater disposal ponds. *Enzyme and Microbial Technology* **26**: 484-490.

Robles, A., Lucas, R., Martinez-Canamero, M., Ben Omar, N., Perez, R. and Galvez, A. (2002). Characterisation of laccase activity produced by the hyphomycete *Chalara* (syn. *Thielavopsis*) *paradoxa* CH32. *Enzyme and Microbial Technology* **31**: 516-522.

Roch, P., Buswell, J. A., Cain, R. B. and Odier, E. (1989). Lignin peroxidase production by strains of *Phanerochaete chrysosporium* grown on glycerol. *Applied Microbiology and Biotechnology* **31**: 587-591.

Rodriguez, E., Pickard, M. A. and Vazquez-Duhalt, R. (1999a). Industrial dye decolorization by laccases from ligninolytic fungi. *Current Microbiology* **38**: 27-32.

Rodriguez, S., Longo, M. A., Cameselle, C. and Sanroman, A. (1999b). Production of manganese peroxidase and laccase in laboratory-scale bioreactors by *Phanerochaete chrysosporium. Bioprocess Engineering* **20**: 531-535.

Roper, J. C., Sarkar, J. M., Dec, J. and Bollag, J.-M. (1995). Enhanced enzymatic removal of chlorophenols in the presence of co-substrates. *Water Research* **29**(12): 2720-2724.

Rosales, E., Couto, S. R. and Sanroman, A. (2002). New uses of food waste: application to laccase production by *Trametes hirsuta*. *Biotechnology Letters* **24**: 701-704.

Ruiz-Duenas, F. J., Martinez, M. J. and Martinez, A. T. (1999a). Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*. *Molecular Microbiology* **31**(1): 223-235.

Ruiz-Duenas, F. J., Martinez, M. J. and Martinez, A. T. (1999b). Heterologous expression of *Pleurotus eryngii* peroxidase confirms its ability to oxidize Mn^{2+} and different aromatic substrates. *Applied and Environmental Microbiology* **65**(10): 4705-4707.

Sakurai, A., Kawamoto, S., Abarca, J. F. and Sakakibara, M. (2002). Peroxidase production by *Coprinus cinereus* using rotating disk contactor. *Biochemical Engineering Journal* **10**: 47-53.

Sakurai, A., Toyoda, S. and Sakakibara, M. (2001). Removal of bisphenol A by polymerization and precipitation method using *Coprinus cinereus* peroxidase. *Biotechnology Letters* **23**: 995-998.

Saparrat, M. C. N., Guillen, F., Arambarri, A. M., Martinez, A. T. and Martinez, M. J. (2002). Induction, isolation, and characterization of two laccases from white rot basidiomycete *Coriolopsis rigida*. *Applied and Environmental Microbiology* **68**(4): 1534-1540.

Sarkar, S., Martinez, A. T. and Martinez, M. J. (1997). Biochemical and molecular characterization of a manganese peroxidase isoenzyme from *Pleurotus ostreatus*. *Biochimica et Biophysica Acta* **1339**: 23-30.

Sawai-Hatanaka, H., Ashikari, T., Tanaka, Y., Asada, Y., Nakayama, T., Minakata, H., Kunishima, N., Fukuyama, K., Yamada, H., Shibano, Y. and Amachi, T. (1995).

Cloning, sequencing, and heterologous expression of a gene coding for *Arthromyces ramosus* peroxidase. *Bioscience, Biotechnology, and Biochemistry* **59**(7): 1221-1228.

Score, A. J., Palfreyman, J. W. and White, N. A. (1997). Extracellular phenoloxidase and peroxidase enzyme production during interspecific fungal interactions. *International Biodeterioration & Biodegradation* **39**(2): 225-233.

Sethuraman, A., Akin, D. E. and Eriksson, K.-E. L. (1999). Production of ligninolytic enzymes and synthetic lignin mineralization by the bird's nest fungus *Cyathus stercoreus*. *Applied Microbiology and Biotechnology* **52**: 689-697.

Shinmen, Y., Asami, S., Amachi, T., Shimizu, S. and Yamada, H. (1986). Crystallization and characterization of an extracellular fungal peroxidase. *Agricultural and Biological Chemistry* **50**(1): 247-249.

Shuttleworth, K. L. and Bollag, J.-M. (1986). Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. *Enzyme and Microbial Technology* **8**: 171-177.

Solomon, E. I., Sundaram, U. M. and Machonkin, T. E. (1996). Multicopper oxidases and oxygenases. *Chemical Reviews* **96**(7): 2563-2605.

Srebotnik, E. and Hammel, K. E. (2000). Degradation of nonphenolic lignin by the laccase/1-hydroxybenzotriazole system. *Journal of Biotechnology* **81**: 179-188.

Steffen, K. T., Hatakka, A. and Hofrichter, M. (2002a). Degradation of humic acids by the litter-decomposing basidiomycete *Collybia dryophila*. *Applied and Environmental Microbiology* **68**(7): 3442-3448.

Steffen, K. T., Hofrichter, M. and Hatakka, A. (2002b). Purification and characterization of manganese peroxidases from the litter-decomposing basidiomycetes *Agrocybe* *praecox* and *Stropharia coronilla*. *Enzyme and Microbial Technology* **30**: 550-555.

Szklarz, G. and Leonowicz, A. (1986). Cooperation between fungal laccase and glucose oxidase in the degradation of lignin derivatives. *Phytochemistry* **25**(11): 2537-2539.

Tanaka, T., Tonosaki, T., Nose, M., Tomidokoro, N., Kadomura, N., Fujii, T. and Taniguchi, M. (2001). Treatment of model soils contaminated with phenolic endocrine-disrupting chemicals with laccase from Trametes sp. in a rotating reactor. *Journal of Bioscience and Bioengineering* **92**(4): 312-316.

Tanaka, T., Yamada, K., Tonosaki, T., Konishi, T., Goto, H. and Taniguchi, M. (2000). Enzymatic degradation of alkylphenols, bisphenol A, synthetic estrogen and phthalic ester. *Water Science and Technology* **42**(7-8): 89-95.

Tekere, M., Zvauya, R. and Read, J. S. (2001). Ligninolytic enzyme production in selected sub-tropical white rot fungi under different culture conditions. *Journal of Basic Microbiology* **41**(2): 115-129.

Thomas, B. R., Yonekura, M., Morgan, T. D., Czapla, T. H., Hopkins, T. L. and Kramer, K. J. (1989). A trypsin-solubilized laccase from pharate pupal integument of the tobacco hornworm, *Manduca sexta*. *Insect Biochemistry* **19**(7): 611-622.

Thurston, C. F. (1994). The structure and function of fungal laccases. *Microbiology* **140**: 19-26.

Tien, M. and Kirk, T. K. (1983). Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. *Science* **221**: 661-663.

Tien, M. and Kirk, T. K. (1984). Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. *Proceedings of the National Academy of Sciences of the United States of America* **81**: 2280-2284.

Tien, M. and Myer, S. B. (1990). Selection and characterization of mutants of *Phanerochaete chrysosporium* exhibiting ligninolytic activity under nutrient-rich conditions. *Applied and Environmental Microbiology* **56**(8): 2540-2544.

Tsujimura, H., Takaya, M., Katano, K., Matsumoto, N., Park, Y. S. and Okabe, M. (1994). Scaleup of peroxidase production by *Arthromyces ramosus* based on analysis of fluid velocity distribution. *Journal of Fermentation and Bioengineering* **77**(6): 650-654.

Tsutsumi, Y., Haneda, T. and Nishida, T. (2001). Removal of estrogenic activities of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. *Chemosphere* **42**: 271-276.

Ullah, E. A., Kadhim, H., Rastall, R. A. and Evans, C. S. (2000). Evalualtion of solid substrates for enzyme production by *Coriolus versicolr*, for use in bioremediation of chlorophenols in aqueous effluents. *Applied Microbiology and Biotechnology* **54**: 832-837.

Ulmer, D., Leisola, M., Puhakka, J. and Fiechter, A. (1983). *Phanerochaete chrysosporium*: Growth pattern and lignin degradation. *European Journal of Applied Microbiology and Biotechnology* **18**: 153-157.

Umezawa, T. and Higuchi, T. (1989). Cleavages of aromatic ring and *β-O*-4 bond of synthetic lignin (DHP) by lignin peroxidase. *FEBS Letters* **242**(2): 325-329.

Valli, K., Wariishi, H. and Gold, M. H. (1992). Degradation of 2,7-dichlorodibenzo-*p*-dioxin by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Journal of Bacteriology* **174**(7): 2131-2137.

Vasconcelos, A. F. D., Barbosa, A. M., Dekker, R. F. H., Scarminio, I. S. and Rezende, M. I. (2000). Optimization of laccase production by *Botryosphaeria* sp. in the presence of veratryl alcohol by the response-surface method. *Process Biochemistry* **35**: 1131-1138.

Velazquez-Cedeno, M. A., Mata, G. and Savoie, J.-M. (2002). Waste-reducing cultivation of *Pleurotus ostreatus* and *Pleurotus pulmonarius* on coffee pulp: Changes in the production of some lignocellulolytic enzymes. *World Journal of Microbiology and Biotechnology* **18**(3): 201-207.

Waldner, R., Leisola, M. S. A. and Fiechter, A. (1988). Comparison of ligninolytic activities of selected white-rot fungi. *Applied Microbiology and Biotechnology* **29**: 400-407.

Wang, Y., Vazquez-Duhalt, R. and Pickard, M. A. (2001). Effect of growth conditions on the production of manganese peroxidase by three strains of *Bjerkandera adusta*. *Canadian Journal of Microbiology* **47**: 277-282.

Wang, Y., Vazquez-Duhalt, R. and Pickard, M. A. (2002). Purification, characterization, and chemical modification of manganese peroxidase from *Bjerkandera adusta* UAMH 8258. *Current Microbiology* **45**: 77-87.

Welinder, K. G. (1992). Superfamily of plant, fungal and bacterial peroxidases. *Current Opinion in Structural Biology* **2**: 388-393.

Yang, Q. X., Yang, M., Pritsch, K., Yediler, A., Hagn, A., Schloter, M. and Kettrup, A. (2003). Decolorization of synthetic dyes and production of manganese-dependent peroxidase by new fungal isolates. *Biotechnology Letters* **25**: 709-713.

Yao, P.-X., Matsumoto, N., Tsujimura, H., Takaya, M. and Toda, K. (1995). Enhanced production of peroxidase in continuous culture of *Arthromyces ramosus* by selective bleeding of mycelium. *Journal of Fermentation and Bioengineering* **79**(4): 307-312.

Young, L. and Yu, J. (1997). Ligninase-catalysed decolorization of synthetic dyes. *Water Research* **31**(5): 1187-1193.

Zhao, J. and Kwan, H. S. (1999). Characterization, molecular cloning, and differential expression analysis of laccase genes from the edible mushroom *Lentinula edodes*. *Applied and Environmental Microbiology* **65**(11): 4908-4913.

Zimmermann, W. (1990). Degradation of lignin by bacteria. *Journal of Biotechnology* **13**: 119-130.

Chapter 4. Screening of *Coprinus* Species for the Production of Extracellular Peroxidase (Part I)*

4.1. Introduction

Phenol, aniline and their derivatives are commonly found in the wastewaters from a variety of industries such as coal conversion, petroleum refining, textile, resins, plastics, wood preservation, and organic chemical manufacturing. Some of the compounds, especially polychlorinated derivatives, are often recalcitrant to conventional biological treatment processes. In addition, many of these aromatic compounds are toxic and some are carcinogenic. An alternative treatment process using enzymes that catalyse oxidation of these compounds was proposed in the early 1980s (Klibanov *et al.*, 1980). In this process, the aqueous phenols and anilines are first oxidised to reactive free radicals or quinones in the presence of a peroxidase and hydrogen peroxide, or a phenol oxidase and molecular oxygen. Subsequently, the reactive products spontaneously polymerise to form water-insoluble polymers that can be removed from the waste streams. Enzymes including peroxidases, laccases and tyrosinases of plant, fungal and microbial origins have been found to be effective in this process (Karam and Nicell, 1997).

A great deal of research has been carried out to characterise the catalytic activities of enzymes in order to optimise the process (Aitken and Irvine, 1989; Ibrahim *et al.*, 1997a; Wright and Nicell, 1999; Ikehata and Nicell, 2000a), to model the kinetics of the removal (Nicell, 1994; Buchanan and Nicell, 1997), to prolong the catalytic life of enzymes during reactions using additives and enzyme immobilization (Sarkar *et al.*, 1989; Nakamoto and Machida, 1992), to assess the residual toxicity of treated solutions (Aitken *et al.*, 1994; Ghioureliotis and Nicell, 2000; Ikehata and Nicell, 2000b). Although these studies have strongly indicated that the enzymatic treatment has a potential for the large-scale application, further research is still required to improve its cost effectiveness, especially regarding the bulk production cost of enzymes.

---

* A version of this chapter has been published. Ikehata, K. and Buchanan, I.D. *Environmental Technology* (2002) **23**(12): 1355-1368.

Among the enzymes studied, horseradish peroxidase (HRP) is commercially available and is one of the best-studied enzymes for the treatment of phenolic wastewaters. However, its production is limited because it is harvested from horseradish roots, which are cultivated mainly for the food industry, growing slowly and requiring large areas for planting. Other enzymes such as soybean peroxidase from soybean hulls (Wright and Nicell, 1999; Kinsley and Nicell, 2000), lignin peroxidases and laccases from wood degrading fungi (Bollag *et al.*, 1988; Aitken *et al.*, 1989), and other fungal peroxidases (Al-Kassim *et al.*, 1994a; Al-Kassim *et al.*, 1994b; Buchanan and Han, 2000) have been investigated as alternatives to HRP. Although some of these enzymes are also commercially available, their production cost is still too high for industrial wastewater treatment. This is mainly because the primary use of these enzymes, especially peroxidases, is aimed at clinical examinations such as immunoassay (Akimoto *et al.*, 1990; Kim *et al.*, 1991) that require highly purified enzymes. Since such high purity is not needed for wastewater treatment (Cooper and Nicell, 1996; Roper *et al.*, 1996), the development of processes for low-cost enzyme production that are strictly designed for wastewater treatment is desired.

In light of this aspect, extracellular fungal peroxidases are likely to have a great potential for application to wastewater treatment because of their relatively simple production and separation procedures compared with other plant and fungal enzymes that are secreted within the tissue of the organisms. Although the applications of *Arthromyces ramosus* peroxidase (ARP) (Al-Kassim *et al.*, 1993; Ibrahim *et al.*, 1997a; Ibrahim *et al.*, 1997b; Buchanan and Han, 2000), *Coprinus macrorhizus* peroxidase (Al-Kassim *et al.*, 1994a; Al-Kassim *et al.*, 1994b) and *Coprinus cinereus* peroxidase (Kauffmann *et al.*, 1999; Masuda *et al.*, 2001) to aqueous phenol treatment have been investigated extensively, the reports on the production of these enzymes for wastewater treatment are still limited. Therefore, the objectives of this study are (1) screening of *Coprinus* and related fungi for the production of extracellular peroxidase, (2) evaluation of the applicability of cultivated fungal peroxidase to the treatment of aqueous phenols, and (3) comparison of the peroxidase with commercially available peroxidases including HRP and ARP. Since it is known that the addition of long chain hydrophilic polymers such as polyethylene glycol (PEG) and chitosan enhances catalytic life of other peroxidases (Nakamoto and Machida, 1992; Ganjidoust *et al.*, 1996; Ibrahim *et al.*, 1997a; Buchanan and Han, 2000; Kinsley and Nicell, 2000), the use of these additives in this fungal peroxidase system was also studied.

4.2. Materials and methods

4.2.1. Materials

4.2.1.1. Fungi

Twenty-two strains of *Coprinus* species were obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH), Alberta, Canada. Three strains of *Coprinus cinereus* were obtained from the Institute for Fermentation, Osaka (IFO), Japan. *Arthromyces ramosus* FERM BP-838 was obtained from the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ibaraki, Japan. The complete list of fungal strains is presented in Table 4.1. Stock cultures of fungi were grown on potato dextrose agar (PDA; Difco, Detroit, Michigan) at 30°C for 5 to 10 days and stored at 4°C.

Table 4.1. List of fungi

Name of fungus	Accession Number of Strain
Arthromyces ramosus	FERM BP-838
Coprinus cinereus	IFO 30114, IFO 30116, IFO 8371, UAMH 4103, UAMH 7907
Coprinus atramentarius	UAMH 7496
Coprinus clastophyllus	UAMH 4104
Coprinus comatus	UAMH 7498
Coprinus domesticus	UAMH 4101
Coprinus kimurae	UAMH 4105
Coprinus lagopus	UAMH 7499
Coprinus micaceus	UAMH 7500
Coprinus psychromorbidus	UAMH 6383
Coprinus sterquilinus	UAMH 4155
Coprinus velox	UAMH 4137
Coprinus sp.	UAMH 358, UAMH 380, UAMH 1211, UAMH3029, UAMH 3261, UAMH 4568, UAMH 6478, UAMH 7251, UAMH 7412, UAMH 7413

4.2.1.2. Enzymes and chemicals

Peroxidases (EC 1.11.1.7) from horseradishes (HRP; RZ = 1.1) and from *Arthromyces ramosus* (ARP; RZ = 2.5), glucose assay (GO) kits, 99% 3,5-dichloro-2-hydroxybenzenesulphonic acid sodium salt (HDCBS; Aldrich brand), and

1.7 *N* acetic acid, and PEG 35 000 (average M.W.; Fluka brand) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). PDA, dextrose, peptone, malt extract and yeast extract manufactured by Difco were purchased from Fisher Scientific Canada (Edmonton, Alberta, Canada). Phenol, 98% 4-aminoantipyrine (4-AAP), 30% w/w hydrogen peroxide, sodium bicarbonate, potassium ferricyanide, sodium hydroxide, sulphuric acid and sodium phosphate monobasic (all ACS certified except for 4-AAP) were purchased from Fisher Scientific Canada. A chitosan sample with a viscosity of 403 centepoise (cps) was kindly provided by Vanson, Redmond, Washington. Stock solution of chitosan was prepared in 5% acetic acid. Ultrapure water produced by Elgastat Maxima Water Purification System (Elga Ltd., High Wycombe, Bucks, England) was used for all reagent preparation.

4.2.1.3. Equipment

A Hewlett-Packard HP 8453 UV-Visible Spectrophotometer was used for the measurement of UV and visible spectra of samples, and colourimetric assays for the activity of peroxidase and concentrations of hydrogen peroxide and phenol. An Ultrospec 2000 UV-Visible Spectrometer (Pharmacia Biotech Ltd., Cambridge, England) was used for glucose assay. Glass and quartz semi-micro cuvettes with a 1 cm optical path and a 1.5 mL volume (Hellma Ltd., Canada) were used in all spectrophotometric measurements. An Orion combination pH electrode with a Fisher Accumet® pH meter model 50 was used for pH measurements. An Immersion Circulator Model C1 (Thermo Haake, Paramus, New Jersey) in combination with a polypropylene water bath was used for glucose assay. A Centra-GP8R Refrigerated Centrifuge (International Equipment Company, Needham Heights, Massachusetts) was used for solid-liquid separation of samples.

4.2.2. Analytical methods

4.2.2.1. Peroxidase activity assay

Peroxidase activity was measured by a colourimetric assay in which 1 mL assay mixture was composed of 500 µL of 20 mM phenol, 250 µL of 9.6 mM 4-AAP, 100 µL of 2 mM hydrogen peroxide (H_2O_2), 0 to 130 µL of 0.1 mM sodium phosphate buffer at pH 7.4 and 20 to 150 µL of biomass-free sample. All assay reagents were prepared in the 0.1 M sodium phosphate buffer at pH 7.4. Immediately after the addition of sample, the formation of quinoneimine dye was monitored using a spectrophotometer at a wavelength of 510 nm at 25°C. One unit (U) of peroxidase activity was defined as the amount of hydrogen peroxide consumed (in µmole) in one minute using an extinction coefficient of 7 100 M^{-1} cm^{-1} based on hydrogen peroxide. In this study the rate of dye formation during the initial 25 seconds was used for the unit calculation because the rate starts to decrease beyond this time in ARP and *Coprinus* peroxidase activity assays.

4.2.2.2. Glucose assay

Concentration of glucose in culture media was determined by a colourimetric assay based on the method described in the glucose (GO) assay kit technical bulletin provided by Sigma (Sigma, 1997). This assay involves the oxidation of D-glucose catalysed by glucose oxidase in the presence of O_2 and water to form D-gluconic acid and H_2O_2, and subsequent oxidation of *o*-dianisidine with H_2O_2 catalysed by peroxidase. The oxidised *o*-dianisidine is transformed to a stable coloured product by the addition of sulphuric acid (H_2SO_4). The intensity of the pink colour measured at 540 nm is proportional to the original glucose concentration. To prepare the assay reagent, one capsule of glucose oxidase/peroxidase reagent was dissolved in 39.2 mL of water and combined with 0.8 mL of 5 g L^{-1} *o*-dianisidine dihydrochloride. At zero time, 2 mL of assay reagent were combined with 1 mL of properly diluted sample in test tube. The test tube was transferred in a water bath in which the temperature was maintained at 37°C. After exactly 30 minutes, 12 *N* H_2SO_4 was added to stop the reaction and to transform the reaction product. The absorbance at 540 nm was measured using a spectrophotometer and converted to glucose concentration using a calibration curve.

4.2.2.3. Hydrogen peroxide assay

Concentration of hydrogen peroxide was measured by a colourimetric assay at 25°C in which 1-mL assay mixture was composed of 500 μL of 18 mM HDCBS, 250 μL of 9.6 mM 4-AAP, 100 μL of 1 mg mL^{-1} HRP solution, 0 – 130 μL of 0.1 mM sodium phosphate buffer at pH 7.4 and 20 – 150 μL of sample. All reagents were prepared in 0.1 mM sodium phosphate buffer at pH 7.4. The colour was allowed to develop for 8 minutes after the reagents were combined and was measured using a spectrophotometer at a wavelength of 510 nm. This absorbance was converted to hydrogen peroxide concentration using a calibration curve.

4.2.2.4. Phenol assay

Concentration of phenol was measured by a colourimetric assay in which the phenol in a sample reacts with 4-AAP in the presence of potassium ferricyanide at 25°C. An aliquot of sample was diluted and brought to a volume of 800 μL with 0.25 mM sodium bicarbonate. Subsequently, 100 μL of 20.8 mM 4-AAP followed by 100 μL of 83.4 mM potassium ferricyanide (both prepared in 0.25 mM sodium bicarbonate) were added to the sample. The absorbance of assay mixture at 510 nm was measured after 6 minutes. When a sample had an absorbance at 510 nm prior to the assay, this absorbance was subtracted from the absorbance measured during the assay. The absorbance at 510 nm due to the reaction between phenol and assay reagents was subsequently converted to phenol concentration using a calibration curve.

4.2.3. Experimental procedures

4.2.3.1. Peroxidase production experiments

Seed culture for liquid fermentation was pre-grown on PDA at 25°C for one to two weeks depending on the fungal strains. Approximately 1 cm^2 of surface mycelia from the seed culture were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of a liquid medium composed of 1% glucose (dextrose), 0.5% peptone, 0.3% yeast extract and 0.3% malt extract (YM broth) and cultivated on a rotary shaker at 175 rpm and 25°C. The initial pH of the culture broth was 6.2 ± 0.1. All cultures were at least triplicated. After an incubation period, ranging from 3 to 15 days, the liquid culture was centrifuged at 3 000 *g* for 30 minutes at 4°C to remove fungal biomass. The biomass was washed with pure water, dried in an oven at 70°C overnight and weighed. Clear supernatant was analysed for peroxidase activity, glucose concentration and pH, then stored at 4°C for future use. Although the activities of lignin degrading enzymes including lignin peroxidase, manganese peroxidase and laccase were also measured using methods described in Pickard *et al.* (1999), none of these enzymes was detected in the culture medium with a significant level of activity.

4.2.3.2. Batch treatment experiments

All batch treatment experiments were carried out in 30-mL borosilicate vials containing 20 mL reaction mixture that was composed of 1.1 mM phenol, 1.5 mM H_2O_2 and various amounts of peroxidase in 0.1 M sodium phosphate buffer at pH 7.0 at 25°C. A concentrated solution of PEG or chitosan, if needed, was also added to the mixture before the reaction was initiated. The final concentration of PEG and chitosan in the reaction mixtures ranged from 25 to 200 mg L^{-1} for each polymer. The pH change due to the addition of chitosan stock solution, which was prepared in 5% acetic acid, was negligible. The reaction was initiated by adding to the reaction mixture an aliquot of peroxidase solution, either in the form of biomass-free supernatant of liquid *Coprinus* culture or as an aqueous solution of HRP or ARP, to yield the desired initial peroxidase activity. The reaction mixture was stirred with a Teflon-coated stir bar propelled by a magnetic stirrer. After 3 hours the reaction solution was centrifuged at 3 000 *g* for 30 minutes to remove suspended materials. Clear supernatant was subjected to the analysis of residual peroxidase activity, concentrations of phenol and H_2O_2, and visible spectra. All treatments were at least duplicated. Two controls were run concurrently in each set of experiments: a reaction mixture without H_2O_2, and one without enzyme. No change in phenol concentration was observed in any of these controls.

4.3. Results and discussion

4.3.1. Production of extracellular fungal peroxidase

4.3.1.1. Screening of fungi

Most of the tested fungi grew well in YM broth at 25°C within 11 days. High extracellular peroxidase activity was found in the liquid cultures of *A. ramosus* FERM BP-838, *Coprinus* sp. UAMH 358 and five strains of *C. cinereus* after 5 and 11 days of growth (Figure 4.1). Although it is not shown in Figure 4.1, *Coprinus* sp. UAMH 380 and *C. lagopus* UAMH 7499 also produced small, but significant amounts of peroxidase after 11 days. Since these two fungi grew very slowly in the liquid medium used, their peroxidase production might be enhanced if the culture conditions were optimised for their growth. No significant peroxidase activity was detected in the rest of the cultures.

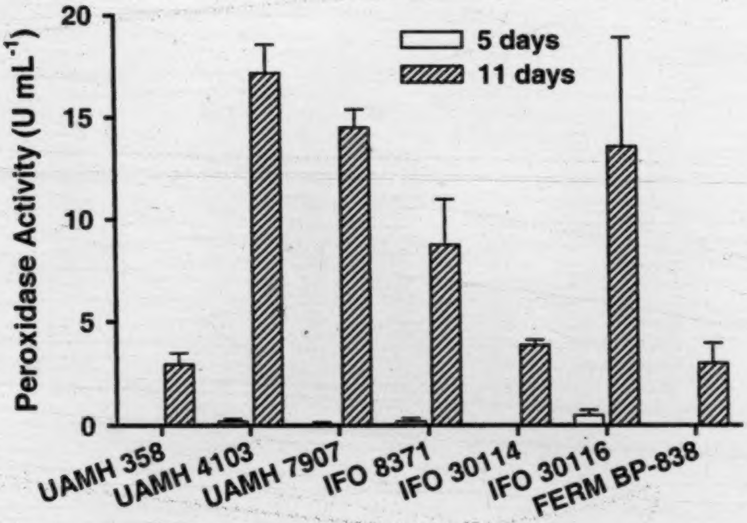


Figure 4.1. Peroxidase activity in the liquid cultures of *Arthromyces ramosus* and *Coprinus* species after 5 and 11 days of growth at 25°C.

The production of extracellular peroxidase has already been reported using *C. cinereus* IFO 30116 (Morita *et al.*, 1988) and IFO 30628 (not used in this study) (Masuda *et al.*, 2001), and *A. ramosus* FERM BP-838 (Shinmen *et al.*, 1986). In this study the production of peroxidase by *C. cinereus* obtained from a different culture collection (UAMH) as well as *C. lagopus* UAMH 7499 and *Coprinus* spp. UAMH 358 and UAMH 380 was demonstrated for the first time. Since it is generally accepted that *C. lagopus* is a close relative of *C. cinereus* (Orton and Watling, 1979; Pukkila and Casselton, 1991), the peroxidase production ability of the former fungus was reasonable. Unlike these fungi, other *Coprinus* species tested including both identified and unidentified ones seemed to have no peroxidase production ability. With information provided by a culture collection, UAMH, and reported in the literature (Webster, 1970; Orton and Watling, 1979; Sigler and Flis, 1998), it can be suggested that the *Coprinus* species that grow in soil (e.g. *C. comatus*), on wood (e.g. *C. micaceus*), or from buried wood (e.g. *C. atramentarius*) may not have an ability to produce peroxidase. In contrast, *Coprinus* species that can grow on dung or manure (*e.g. C. cinereus* and *Coprinus* sp. UAMH 358) may have the ability to produce peroxidase, although another coprophilous (dung-loving) fungus *C. sterquilinus* UAMH 4155 did not show this property. Although little is known about the relationship between the peroxidase production and ecology of the fungus, knowing the ecological and physiological roles of peroxidase for *Coprinus* species may be useful to optimise growth conditions for enzyme production. Therefore, peroxidase production of additional *Coprinus* species including some coprophilous ones, which have been isolated from urea-treated soil in local coniferous woods, is currently under investigation in the authors' laboratories.

Under the tested culture conditions, the *Coprinus* species examined in this study did not produce lignin peroxidase, manganese peroxidase or laccase, which are known to be responsible for lignin degradation by a variety of white-rot fungi such as *Phanerochaete chrysosporium* (Huynh and Crawford, 1985), *Trametes versicolor* (Bollag and Leonowicz, 1984) and *Bjerkandera adusta* (Kimura *et al.*, 1991). Although ARP, which is essentially an identical protein to CIP (Kjalke *et al.*, 1992), shares more than 40% of amino acid sequence with lignin and manganese peroxidases, the substrate specificities of these two types of fungal peroxidases are different from one another (Sawai-Hatanaka *et al.*, 1995). It can be suggested that the peroxidase secreted by *Coprinus* species did not have lignin degradation ability because these fungi are mostly terrestrial or coprophilous and need little ligninolytic activity in their natural habitats.

Using Ryan's procedure (Ryan, 1959) for multiple comparisons of seven superior cultures shown in Figure 4.1 with a nominal significant level, α', of 0.005, the peroxidase activity in the culture of *C. cinereus* UAMH 4103 (17.1 ± 1.4 U mL^{-1}) was significantly higher than that in the culture of *Coprinus* sp. UAMH 358, *C. cinereus* IFO 8371, IFO 30114, and *A. ramosus* FERM BP-838 after 11 days of growth. Similarly, the peroxidase activity in the cultures of *C. cinereus* UAMH 7907 and IFO 30116 (14.5 ± 0.9 U mL^{-1} and 13.6 ± 5.3 U mL^{-1}, respectively) were significantly higher than that in the cultures of UAMH 358, IFO 30114 and FERM BP-838 after 11 days of growth. No significant difference was found in other combinations of fungal strains.

4.3.1.2. Time course of peroxidase production

Based on the analysis shown above, three strains of *C. cinereus* including UAMH 4103, UAMH 7907 and IFO 30116 were selected for more detailed study on their growth and corresponding extracellular peroxidase production. The changes in fungal biomass, glucose concentration, peroxidase activity and pH in YM broth were monitored over a 15-day period after the inoculation. After a short acclimation period, the biomass increased rapidly, until glucose was completely consumed, and decreased slightly thereafter (Figure 4.2). Peroxidase activity was detected soon after the lag period and reached its peak 6 days later. While the peroxidase production was likely to be correlated with the accumulation of fungal biomass, the activity declined more rapidly than the biomass after the complete depletion of glucose. The pH of liquid culture was initially 6.1 and decreased slightly for 6 days. Thereafter it increased gradually to 8 by the day 15 in all cultures. Although the pH-dependent stability of CIP has not been documented yet, some of the enzyme might be inactivated due to the increasing pH in the culture. Thus, it can be suggested that controlling pH of culture media during fermentation may be needed to maximise the enzyme yields.

There was no significant difference in the peak activity of peroxidase among the three strains of *C. cinereus* shown in Figure 4.2 ($p = 0.27$ in one way ANOVA test). However, *C. cinereus* UAMH 7907 was discarded for further investigations because of its slower rate to reach maximum peroxidase activity than the others (Figure 4.2). Further improvement of peroxidase production using two strains of *C. cinereus* UAMH 4103 and

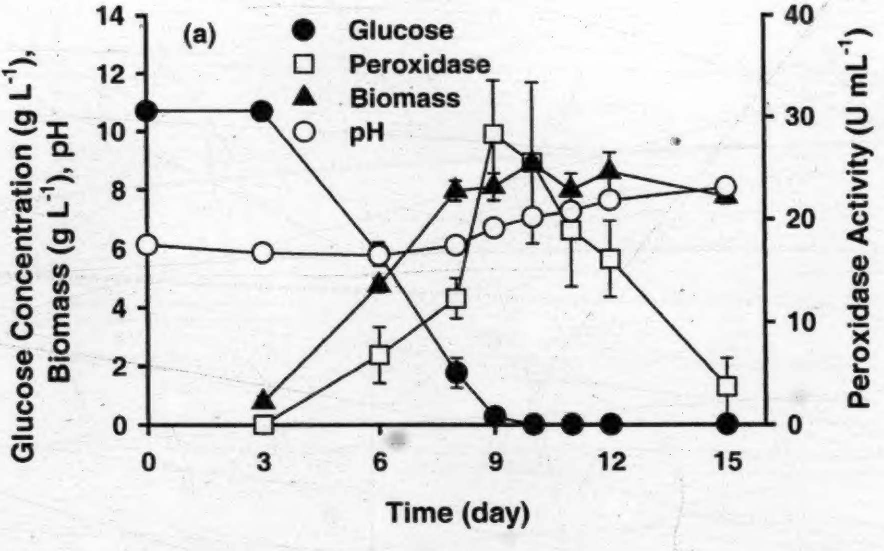
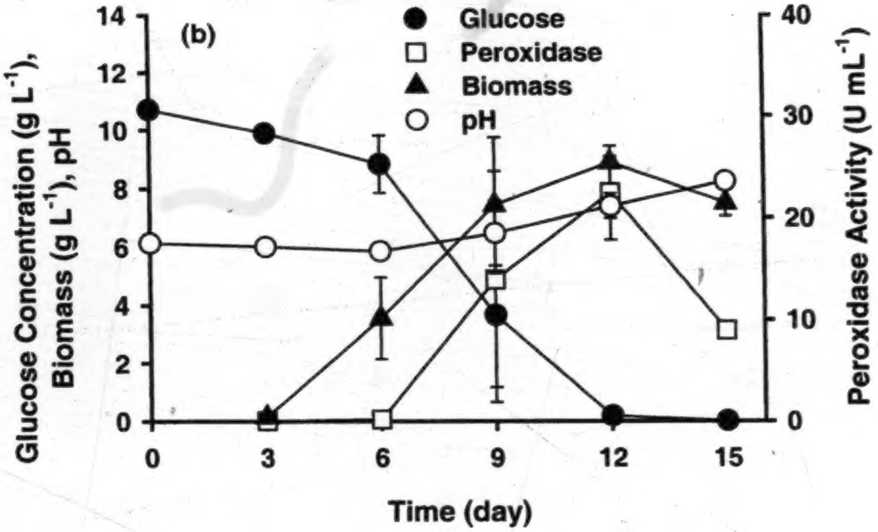


Figure 4.2. Growth of *C. cinereus* and its production of peroxidase at 25°C: (a) UAMH 4103, (b) UAMH 7907 and (c) IFO 30116 (See next page).



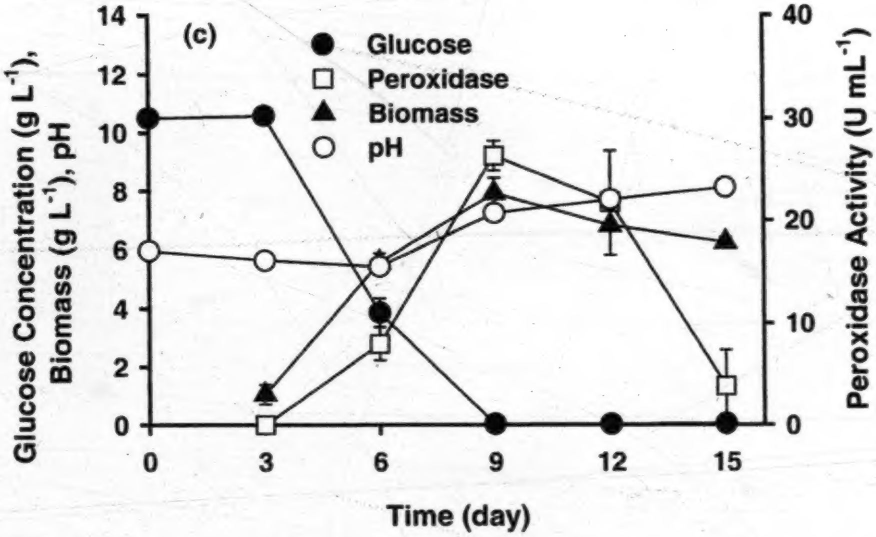


Figure 4.2. Growth of *C. cinereus* and its production of peroxidase at 25°C: (a) UAMH 4103, (b) UAMH 7907 and (c) IFO 30116 (continued).

IFO 30116 is currently being attempted by means of optimisation of growth conditions. Semi-continuous and continuous production of peroxidase in a bioreactor is also under investigation.

4.3.2. Treatment of aqueous phenol with **Coprinus cinereus** *peroxidase*

The supernatant of centrifuged liquid culture of *C. cinereus* UAMH 4103 was used as a crude stock solution of *C. cinereus* peroxidase (CIP) without further purification. The crude enzyme retained at least 90% of initial activity after two weeks of storage at 4°C. There was no marked difference in phenol transformation efficiency, mM phenol transformed per U mL^{-1} of peroxidase provided, among the peroxidases from the cultures of various strains of *C. cinereus* (data not shown).

4.3.2.1. Effects of initial peroxidase concentration and protective additives

In order to quantify the effectiveness of cultivated enzyme on the treatment of aqueous phenol, 1.1 mM phenol solution was treated with various amounts of CIP in 0.1 M sodium phosphate buffer at pH 7.0. Based on preliminary test results, excess H_2O_2 (1.5 mM) was provided to ensure the transformation of phenol was only limited by the availability of enzyme. The effects of high molecular weight PEG and chitosan (poly-D-glucosamine) on the enzymatic treatment were also examined. These two polymers are known to reduce the rate of inactivation of other peroxidases in the presence of aromatic substrates and H_2O_2 (Nakamoto and Machida, 1992; Al-Kassim *et al.*, 1993; Ganjidoust *et al.*, 1996; Kinsley and Nicell, 2000; Wagner and Nicell, 2001). PEG with an average molecular weight of 35 000 Da was selected to use based on a report of Kinsley and Nicell (Kinsley and Nicell, 2000).

Preliminary experiments showed that the addition of PEG was effective to prolong the catalytic activity of CIP at the concentration of as low as 25 mg L^{-1}, and no further improvement or deterioration was observed when excess PEG was applied. On the other hand, while chitosan was also effective at low concentrations (25 mg L^{-1}), the effect gradually diminished with increasing concentration of chitosan and became even detrimental at the concentration of 200 mg L^{-1}. Based on these facts, therefore, the concentrations of PEG and chitosan were fixed to be 200 mg L^{-1} and 25 mg L^{-1}, respectively, in this study. No change in phenol concentration was observed due to the sole addition of these polymers.

After 3 hours CIP was completely inactivated in all reaction mixtures that contained both phenol and H_2O_2, which implied that the enzymatic reaction was completed. No significant loss of CIP activity was observed in the controls containing only phenol during the same time period. The mechanisms that might be responsible for the inactivation of peroxidase during the enzymatic treatment were: 1) haem destruction and covalent modification of peroxidase by free radicals arising from catalytic reactions between aromatic substrates and peroxidase (Chang *et al.*, 1999); 2) entrapment of enzyme by growing polymeric products (Nakamoto and Machida, 1992); and 3) oxidation of enzyme by hydrogen peroxide (Arnao *et al.*, 1990).

Figure 4.3 shows the residual phenol concentration after the CIP-catalysed treatment as a function of initial enzyme activity with and without additives. About 1.1 U mL^{-1} of initial CIP was sufficient to achieve complete transformation of 1.1 mM phenol in the absence of additives. The required initial activity of CIP needed for complete transformation was reduced to about 0.9 U mL^{-1} and 0.8 U mL^{-1} in the presence of chitosan and PEG, respectively. As shown in Figure 4.4, the ratio of the phenol transformed and H_2O_2 consumed was close to 1 both in the presence and in the absence of additives where limiting amounts of enzyme was applied. This, combined with the results shown in Figure 4.3, implies that the addition of either of these polymers extends the catalytic life of the enzyme without affecting the stoichiometry of the reaction.

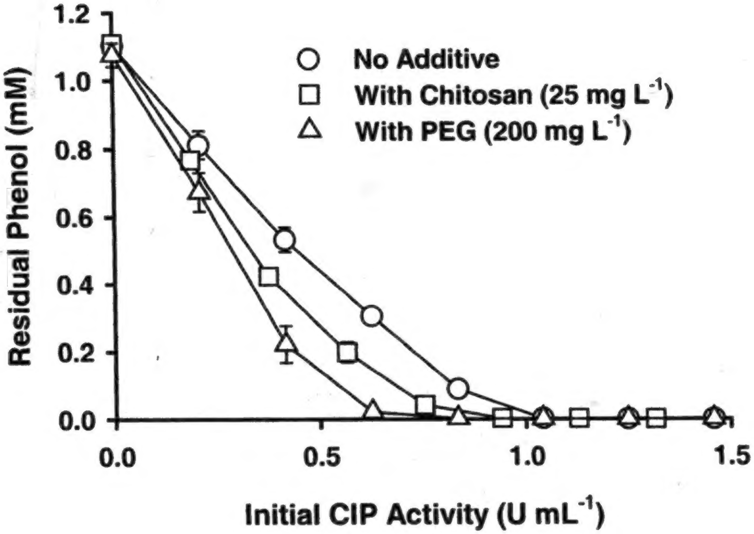


Figure 4.3. Residual phenol as a function of initial CIP activity: $[Phenol]_0 = 1.1$ mM, $[H_2O_2]_0 = 1.5$ mM, in 0.1 M sodium phosphate buffer at pH 7 at 25°C.

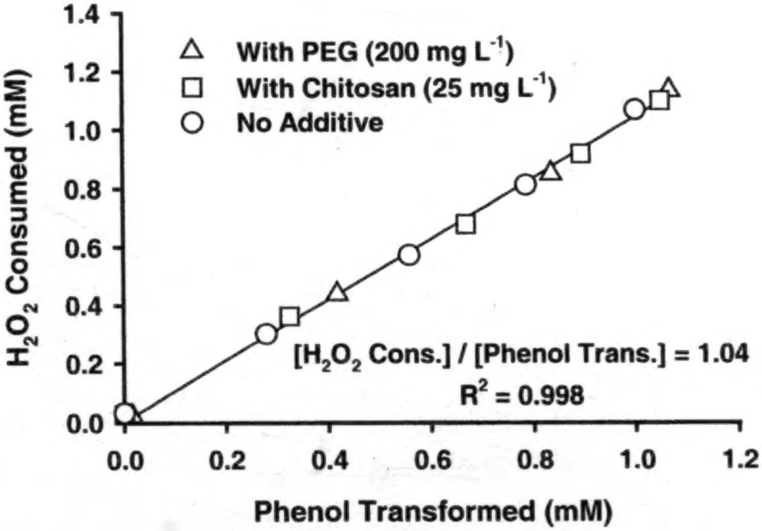


Figure 4.4. Phenol transformed as a function of the H_2O_2 consumed with various amounts of CIP: $[Phenol]_0 = 1.1$ mM, $[H_2O_2]_0 = 1.5$ mM, in 0.1 M sodium phosphate buffer at pH 7 at 25°C.

Although the transformed phenol spontaneously precipitated in the reaction solution to which limiting amounts of CIP were provided, significant amounts of coloured products, which did not readily precipitate, formed when excess enzyme was added. The coloured materials could not be removed either by centrifugation or by membrane filtration (pore size 2.5 μm). As shown in Figure 4.5, the more excessive CIP provided, the more coloured products formed. Although the formation of coloured products occurred in the presence and in the absence of both additives, it was partially suppressed when chitosan was added. This is probably due to the nature of chitosan molecules that possess amino groups that act as reactive adsorbents to reactive phenolic species (Sun *et al.*, 1992; Wada *et al.*, 1995; Ikehata and Nicell, 2000b). A broad peak at the wavelength of 410 nm in the visible spectra of the reaction solution was observed

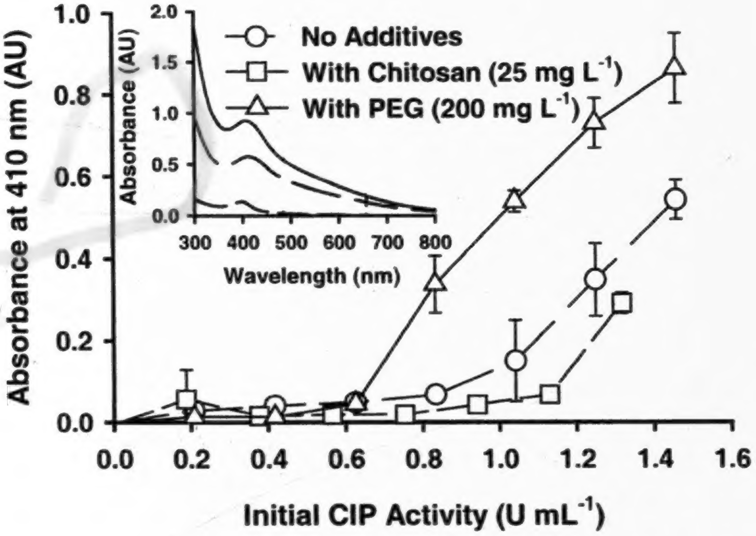


Figure 4.5. Colour formation as a function of initial CIP activity: $[\mathrm{Phenol}]_0 = 1.1$ mM, $[H_2O_2]_0 = 1.5$ mM, in 0.1 M sodium phosphate buffer at pH 7 at 25°C. Inset, the visible spectrum of reaction solutions: $[\mathrm{CIP}]_0 = 1.4$ U mL^{-1}.

after the treatment with excess CIP as shown in the inset of Figure 4.5. This peak absorbance is presumably attributed to the π conjugation of lengthened aromatic rings of phenolic oligomers that are products of spontaneous coupling reactions of phenolic radicals (Yu *et al.*, 1994). These oligomers were likely to be formed due to high initial enzyme/phenol ratio. Although the colour in the reaction solution still persisted after an overnight agitation, the peak absorbance at 410 nm diminished from the spectrum and resulted in a broad shoulder extending from the UV range (data not shown). This implies non-enzymatic reactions of phenolic oligomers had occurred in the reaction solution. More detailed analysis of the reaction products is currently underway.

4.3.2.2. Comparison of peroxidases

Under the reaction conditions described previously, a series of experiments was conducted to compare the performance of CIP with those of horseradish peroxidase (HRP), which has been studied for enzymatic treatment of phenols for years, and *Arthromyces ramosus* peroxidase (ARP), which is known to be essentially an identical protein to CIP (Kjalke *et al.*, 1992). When no additive was used, about 4.5 U mL^{-1} of HRP was needed to achieve complete transformation of 1.1 mM phenol, and more than half of initial phenol remained after the treatment with 4 U mL^{-1} of ARP. However, the initial activity of HRP and ARP required for complete transformation of phenol were reduced to 0.3 U mL^{-1} and 0.8 U mL^{-1}, respectively, in the presence of PEG, and to about 3 U mL^{-1} for both HRP and ARP in the presence of chitosan. The ratios of phenol transformed and H_2O_2 consumed during the HRP- and ARP-catalysed reactions were both 1.0, which is the same as that during the CIP-catalysed reaction. Colour formation similar to that shown in Figure 4.5 was also observed when excess HRP or ARP was added to the reaction solution.

Figure 4.6 shows the amount of phenol transformed with limiting amounts of peroxidases (0.4 U mL^{-1}) in the presence and in the absence of additives. It was shown that in the absence of additives, 2.7 and 4.4 times more phenol was transformed with CIP than with HRP and ARP, respectively. In the presence of PEG, however, the extent of transformation of phenol with CIP and ARP were nearly the same, and 0.4 U mL^{-1} of HRP was sufficient to transform all of the phenol. There is no significant difference in the amount of transformed phenol in the presence of chitosan for all three peroxidases.

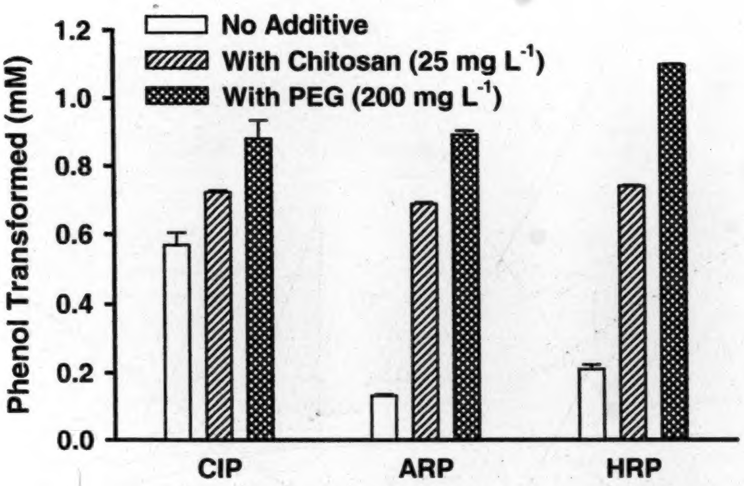


Figure 4.6. Comparison of three enzymes on the phenol transformation: $[Phenol]_0 = 1.1$ mM, $[H_2O_2]_0 = 1.5$ mM, $[peroxidase]_0 = 0.4$ U mL^{-1}, in 0.1 M sodium phosphate buffer at pH 7 at 25°C.

As compared with ARP, the CIP used in this study could transform aqueous phenol with much higher efficiency in the absence of protective additives. Masuda *et al.* (2001) also reported the similar effect of crude CIP that was 6 times more efficient in removing phenol than purified CIP. Since the CIP stock solution was crude supernatant of liquid culture of *C. cinereus*, many soluble by-products of fungal metabolism were also introduced into the phenol-H_2O_2 reaction mixture. The by-products seemingly comprise a variety of compounds ranging from low molecular weight organic and inorganic compounds to macromolecules such as proteins and polysaccharides. It may be that the macromolecules in the supernatant have some protective effects similar to those of PEG and chitosan on CIP, which results in the higher transformation efficiency shown in Figure 4.6. In the presence of additives, on the other hand, the superiority of crude CIP to purified ARP faded away, which implied that the protective effects of polymer additives were limited to the same levels that pure enzyme and the additives could achieve. Similar observation was reported where crude horseradish extract in combination with PEG was examined for the treatment of foundry wastewater (Cooper and Nicell, 1996). Since the actual components contributing to the protection of enzyme are still unknown, more detailed analysis is needed on this issue.

The crude CIP was more effective in transforming aqueous phenol than purified HRP in the absence of additives. This is also likely due to the protective effect of other constituents remaining in the CIP culture broth. As already reported in literature (Nakamoto and Machida, 1992; Nicell *et al.*, 1995; Wu *et al.*, 1997), however, the efficiency of HRP in transforming phenol was greatly enhanced in the presence of PEG and well surpassed that of CIP. The addition of chitosan also enhanced the phenol transformation efficiency of HRP moderately, as has also been reported (Ganjidoust *et al.*, 1996). While the presence of soluble by-products in the crude CIP solution may be beneficial for wastewater treatment because of their protective effects against enzyme inactivation, the unnecessary introduction of an additional organic load is not desirable. A solution of the crude enzyme containing 1 U mL^{-1} of active CIP exerts 170 mg L^{-1} COD (chemical oxygen demand) as compared to 10 mg L^{-1} COD for a solution of purified ARP with the same activity. The use of PEG also increases organic loading by 1.0 mg L^{-1} COD for every mg L^{-1} PEG added. Although the concentration of PEG can be optimised, its use in conjunction with purified enzyme would in most cases result in the addition of an organic load comparable to that added by the crude enzyme solution. Therefore, the balance of organic loading and protective effect should be carefully controlled in all cases. Depending on the situation, the partial purification and concentration of enzyme using ultrafiltration may be recommended to remove smaller organic and inorganic molecules and reduce the volume of stock enzyme solution for handling. In light of this perspective, treatment of real phenolic wastewater samples using cultivated peroxidase is currently being investigated.

4.4. Conclusion

Three strains of *C. cinereus* including UAMH 4103, UAMH 7907 and IFO 30116 were found to produce large amounts of extracellular peroxidase that was very effective for the removal of aqueous phenol. Other *Coprinus* species including *C. lagopus* UAMH 7499 and *Coprinus* spp. UAMH 358 and UAMH 380 also produced modest amounts of peroxidase. Further improvement of peroxidase production will be attempted through optimisation of growth conditions and testing of more *Coprinus* fungi. Crude CIP cultivated from liquid culture of *C. cinereus* UAMH 4103 showed better phenol transformation efficiency than purified ARP, which is known to be very similar to CIP. It was likely due to the protective effect of soluble by-products of fungal metabolism such as polycarbohydrates and proteins against inactivation of CIP during the catalytic transformation of phenol. Although the addition of PEG and chitosan could improve the efficiency of CIP-catalysed phenol transformation further, the extent was limited to the same levels that pure enzyme (ARP) and the additives could achieve. As compared with HRP, crude CIP was superior in terms of phenol transformation efficiency in the absence of additives but inferior in the presence of PEG. While the presence of the soluble by-products of fungal culture may be beneficial for wastewater treatment because of their protective effects on peroxidase, the organic loads due to the addition of crude enzyme solution should be carefully controlled.

References

Aitken, M. D. and Irvine, R. L. (1989). Stability testing of ligninase and Mn-peroxidase from *Phanerochaete chrysosporium*. *Biotechnology and Bioengineering* **34**: 1251-1260.

Aitken, M. D., Massey, I. J., Chen, T. and Heck, P. E. (1994). Characterization of reaction products from the enzyme catalyzed oxidation of phenolic pollutants. *Water Research* **28**(9): 1879-1889.

Aitken, M. D., Venkatadri, R. and Irvine, R. L. (1989). Oxidation of phenolic pollutant by a lignin degrading enzyme from the white-rot fungus *Phanerochaete chrysosporium*. *Water Research* **23**(4): 443-450.

Akimoto, K., Sinmen, Y., Sumida, M., Asami, S., Amachi, T., Yoshizumi, H., Saeki, Y., Shimizu, S. and Yamada, H. (1990). Luminol chemiluminescence reaction catalyzed by a microbial peroxidase. *Analytical Biochemistry* **189**: 182-185.

Al-Kassim, L., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1993). Aromatic removal from water by *Arthromyces ramosus* peroxidase. in *Plant Peroxidases: Biochemistry and Physiology*. Welinder, K. G., Rasmussen, S. K., Penel, C. and Greppin, H., Editors, University of Geneva, Geneva: 197-200.

Al-Kassim, L., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1994a). Optimization of phenol removal by a fungal peroxidase from *Coprinus macrorhizus* using batch, continuous, and discontinuous semibatch reactors. *Enzyme and Microbial Technology* **16**: 120-124.

Al-Kassim, L., Taylor, K. E., Nicell, J. A., Bewtra, J. K. and Biswas, N. (1994b). Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxide from *Coprinus macrorhizus* in batch reactors. *Journal of Chemical Technology and Biotechnology* **61**: 179-182.

Arnao, M. B., Acosta, M., DelRio, J. A. and Garcia-Canovas, F. (1990). Inactivation of peroxidase by hydrogen peroxide and its protection by a reductant agent. *Biochimica et Biophysica Acta* **1038**: 85-89.

Bollag, J.-M. and Leonowicz, A. (1984). Comparative studies of extracellular fungal laccases. *Applied and Environmental Microbiology* **48**(4): 849-854.

Bollag, J.-M., Shuttleworth, K. L. and Anderson, D. H. (1988). Laccase-mediated detoxificaion of phenolic compounds. *Applied and Environmental Microbiology* **54**(12): 3086-3091.

Buchanan, I. D. and Han, Y. S. (2000). Assessment of the potential of *Arthromyces ramosus* peroxidase to remove phenol from industrial wastewaters. *Environmental Technology* **21**: 545-552.

Buchanan, I. D. and Nicell, J. A. (1997). Model development for horseradish peroxidase catalyzed removal of aqueous phenol. *Biotechnology and Bioengineering* **54**(3): 251-261.

Chang, H. C., Holland, R. D., Bumpus, J. A., Churchwell, M. I. and Doerge, D. R. (1999). Inactivation of *Coprinus cinereus* peroxidase by 4-chloroaniline during turnover: comparison with horseradish peroxidase and bovine lactoperoxidase. *Chemico-Biological Interactions* **123**: 197-217.

Cooper, V. A. and Nicell, J. A. (1996). Removal of phenols from a foundry wastewater using horseradish peroxidase. *Water Research* **30**(4): 954-964.

Ganjidoust, H., Tatsumi, K., Wada, S. and Kawase, M. (1996). Role of peroxidase and chitosan in removing chlorophenols from aqueous solution. *Water Science and Technology* **34**(10): 151-159.

Ghioureliotis, M. and Nicell, J. A. (2000). Toxicity of soluble products from the peroxidase-catalysed polymerization of substituted phenolic compounds. *Journal of Chemical Technology and Biotechnology* **75**: 98-106.

Huynh, V.-B. and Crawford, R. L. (1985). Novel extracellular enzymes (ligninases) of *Phanerochaete chrysosporium*. *FEMS Microbiology Letters* **28**: 119-123.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997a). Optimization of the removal of phenol from wastewater catalyzed by *Arthromyces ramosus* peroxidase. *CSCE/ACSE Environ. Eng. Conf.*, Edmonton, Alberta, Canada, Canadian Society for Civil Engineering, Montreal, Quebec. **2**: 1503-1510.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997b). Removal of phenol from industrial wastewater using *Arthromyces ramosus* peroxidase in a continuous flow system. *Proc. of the 52nd Indust. Waste Conf.*, Purdue

University, West Lafayette, Indiana, Ann Arbor Press, Ann Arbor, Michigan: 271-277.

Ikehata, K. and Nicell, J. A. (2000a). Characterization of tyrosinase for the treatment of aqueous phenols. *Bioresource Technology* **74**(3): 191-199.

Ikehata, K. and Nicell, J. A. (2000b). Color and toxicity removal following tyrosinase-catalyzed oxidation of phenols. *Biotechnology Progress* **16**(4): 533-540.

Karam, J. and Nicell, J. A. (1997). Potential application of enzymes in waste treatment. *Journal of Chemical Technology and Biotechnology* **69**: 141-153.

Kauffmann, C., Petersen, B. R. and Bjerrum, M. J. (1999). Enzymatic removal of phenols from aqueous solutions by *Coprinus cinereus* peroxidase and hydrogen peroxide. *Journal of Biotechnology* **73**: 71-74.

Kim, B. B., Pisarev, V. V. and Egorov, A. M. (1991). A comparative study of peroxidases from horse radish and *Arthromyces ramosus* as labels in luminol-mediated chemiluminescent assays. *Analytical Biochemistry* **199**: 1-6.

Kimura, Y., Asada, Y., Oka, T. and Kuwahara, M. (1991). Molecular analysis of a *Bjerkandera adusta* lignin peroxidase gene. *Applied Microbiology and Biotechnology* **35**: 510-514.

Kinsley, C. and Nicell, J. A. (2000). Treatment of aqueous phenol with soybean peroxidase in the presence of polyethylene glycol. *Bioresource Technology* **13**(2): 139-146.

Kjalke, M., Andersen, M. B., Schneider, P., Christensen, B., Schulein, M. and Welinder, K. G. (1992). Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. *Biochimica* *et Biophysica Acta* **1120**: 248-256.

Klibanov, A. M., Alberti, B. N., Morris, E. D. and Felshin, L. M. (1980). Enzymatic removal of toxic phenols and anilines from waste waters. *Journal of Applied Biochemistry* **2**: 414-421.

Masuda, M., Sakurai, A. and Sakakibara, M. (2001). Effect of reaction conditions on phenol removal by polymerization and precipitation using *Coprinus cinereus* peroxidase. *Enzyme and Microbial Technology* **28**: 295-300.

Morita, Y., Yamashita, H., Mikami, B., Iwamoto, H., Aibara, S., Terada, M. and Minami, J. (1988). Purification, crystallization, and characterization of peroxidase from *Coprinus cinereus*. *Journal of Biochemistry* **103**(4): 693-699.

Nakamoto, S. and Machida, N. (1992). Phenol removal from aqueous solutions by peroxidase-catalyzed reaction using additives. *Water Research* **26**(1): 49-54.

Nicell, J. A. (1994). Kinetics of horseradish peroxidase-catalyzed polymerization and precipitation of aqueous 4-chlorophenol. *Journal of Chemical Technology and Biotechnology* **60**: 203-215.

Nicell, J. A., Saadi, K. W. and Buchanan, I. D. (1995). Phenol polymerization and precipitation by horseradish peroxidase enzyme and an additive. *Bioresource Technology* **54**: 5-16.

Orton, P. D. and Watling, R. (1979). Coprinaceae Part 1: Coprinus. in *British Fungus Flora Agarics and Boleti*. Henderson, D. M., Orton, P. D. and Watling, R., Editors. Royal Botanic Garden, Edinburgh, Her Majesty's Stationery Office: 7-125.

Pickard, M. A., Vandertol, H., Roman, R. and Vazquez-Duhalt, R. (1999). High production of ligninolytic enzymes from white rot fungi in cereal bran liquid medium. *Canadian Journal of Microbiology* **45**: 627-631.

Pukkila, P. J. and Casselton, L. A. (1991). Molecular genetics of the agaric *Coprinus cinereus*. in *More Gene Manipulations in Fungi*. Bennett, J. W. and Lasure, L. L., Editors. Sandiego, California, Academic Press, Inc.: 126-150.

Roper, J. C., Dec, J. and Bollag, J.-M. (1996). Biodegradation and bioremediation: Using minced horseradish roots for the treatment of polluted waters. *Journal of Environmental Quality* **25**: 1242-1247.

Ryan, T. A. (1959). Multiple comparisons in psychological research. *Psychological Bulletin* **56**(1): 26-47.

Sarkar, J. M., Leonowicz, A. and Bollag, J.-M. (1989). Immobilization of enzymes on clays and soils. *Soil Biology and Biochemistry* **21**(2): 223-230.

Sawai-Hatanaka, H., Ashikari, T., Tanaka, Y., Asada, Y., Nakayama, T., Minakata, H., Kunishima, N., Fukuyama, K., Yamada, H., Shibano, Y. and Amachi, T. (1995). Cloning, sequencing, and heterologous expression of a gene coding for *Arthromyces ramosus* peroxidase. *Bioscience, Biotechnology, and Biochemistry* **59**(7): 1221-1228.

Shinmen, Y., Asami, S., Amachi, T., Shimizu, S. and Yamada, H. (1986). Crystallization and characterization of an extracellular fungal peroxidase. *Agricultural and Biological Chemistry* **50**(1): 247-249.

Sigler, L. and Flis, A. L. (1998). *University of Alberta Microfungus Collection and Herbarium Catalogue of Strains*, University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Also at: http://www.devonian.ualberta.ca/uamh.

Sigma (1997). *Sigma Technical Bulletin No. GOB-2 (5-97)*. St. Louis, Missouri.

Sun, W.-Q., Payne, G. F., Moas, M. S. G. L., Chu, J. H. and Wallance, K. K. (1992). Tyrosinase reaction/chitosan adsorption for removing phenols from wastewater. *Biotechnology Progress* **8**(3): 179-186.

Wada, S., Ichikawa, H. and Tatsumi, K. (1995). Removal of phenols and aromatic amines from wastewater by a combination treatment with tyrosinase and coagulant. *Biotechnology and Bioengineering* **45**(4): 304-309.

Wagner, M. and Nicell, J. A. (2001). Peroxidase-catalyzed removal of phenols from a petroleum refinery wastewater. *Water Science and Technology* **43**(2): 253-260.

Webster, J. (1970). *Introduction to Fungi*. Cambridge, Cambridge University Press.

Wright, H. and Nicell, J. A. (1999). Characterization of soybean peroxidase for the treatment of aqueous phenol. *Bioresource Technology* **70**(1): 69-79.

Wu, Y., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997). Comparison of additives in the removal of phenolic compounds by peroxidase-catalyzed polymerization. *Water Research* **31**(11): 2699-2704.

Yu, J., Taylor, K. E., Zou, H., Biswas, N. and Bewtra, J. K. (1994). Phenol conversion and dimeric intermediates in horseradish peroxidase-catalyzed phenol removal from water. *Environmental Science and Technology* **28**(12): 2154-2160.

Chapter 5. Screening of *Coprinus* Species for the Production of Extracellular Peroxidase (Part II)*

5.1. Introduction

Peroxidases are a group of haem containing oxidoreductases that catalyse the reduction of peroxide such as hydrogen peroxide and the oxidation of a variety of organic and inorganic compounds. The term peroxidase represents a group of specific enzymes such as NAD peroxidase (EC 1.11.1.1), glutathione peroxidase (EC 1.11.1.9) and iodide peroxidase (EC 1.11.1.8), as well as a group of non-specific enzymes that are simply known as peroxidases (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7). Typical non-specific peroxidases such as horseradish peroxidase (HRP) catalyse the dehydrogenation of varieties of phenols and aromatic amines in the presence of hydrogen peroxide. Peroxidase is a valuable biocatalyst with a range of commercial applications including clinical assays (Allain *et al.*, 1974), chemical and biochemical sensing (Smith *et al.*, 2002), chemical synthesis (Dordick, 1992), and bioremediation and wastewater treatment (Klibanov *et al.*, 1980; Nicell *et al.*, 1993). Enzymatic removal of phenolic compounds and aromatic amines from industrial wastewater is one of the potential applications of peroxidase and has been studied extensively in recent years. However, this process requires large amounts of enzyme, and the high cost associated with enzyme production still hinders its large-scale application.

---

* A version of this chapter (by Ikehata, K., Buchanan, I.D. and Smith, D.W.) has been submitted to *Canadian Journal of Microbiology* in May 2003.

A few fungi including *Arthromyces ramosus* and *Coprinus cinereus* (= *C. macrorhizus*) are known to produce non-specific extracellular peroxidase (Shinmen *et al.*, 1986; Morita *et al.*, 1988), and the enzymes from these fungi (ARP and CIP, respectively) have been investigated as alternatives to HRP in various applications such as luminol chemiluminescence assay (Akimoto *et al.*, 1990; Kim *et al.*, 1991) and phenolic wastewater treatment (Al-Kassim *et al.*, 1994b; Buchanan and Han, 2000; Masuda *et al.*, 2001; Villalobos and Buchanan, 2002). It is known that ARP and CIP do not have ligninolytic activities unlike other well-known fungal peroxidases including lignin peroxidase (LiP) and manganese peroxidase (MnP) (Sawai-Hatanaka *et al.*, 1995). In addition to *A. ramosus* and *C. cinereus*, the production of extracellular peroxidase by other *Coprinus* species such as *C. lagopus* was also demonstrated recently (Ikehata and Buchanan, 2002). These fungal enzymes have several advantages for large-scale production such as fast growth and production in bioreactor, which is relatively simple to scale up, and easy separation due to their extracellular nature.

Coprinus species are basidiomycetes forming mushrooms on litter, soil or decaying wood, and commonly found in many parts of the world (Orton and Watling, 1979). In our previous study, only eight strains of *Coprinus* species were found to produce extracellular peroxidase among the 25 strains evaluated (Ikehata and Buchanan, 2002). Although it was speculated that the ecological and/or taxonomic characteristics might provide a clue, the reasons for the peroxidase production by some fungi are still uncertain. In order to clarify this issue as well as to identify good peroxidase producers for large production and characterisation of the fungal peroxidase for wastewater treatment, an additional screening of *Coprinus* species was conducted and is reported here.

5.2. Materials and methods

5.2.1. Fungi

Nine strains of identified *Coprinus* species including *C. echinosporus* NBRC 30630 and NBRC 30631, *C. lagopides* NBRC 30120, *C. macrocephalus* NBRC 30117, *C. neolagopus* NBRC 30476 and NBRC 32947, *C. phlyctidosporus* NBRC 30478 and NBRC 32946 and *C. radiatus* NBRC 30118 were obtained from the Institute for Fermentation, Osaka, Japan. Please note that the above fungi are currently distributed by NITE Biological Resources Center, National Institute of Technology and Evaluation (NBRC), Chiba, Japan. Four strains of unidentified *Coprinus* species (UAMH 10065, UAMH 10066, UAMH 10067 and 074) recently isolated from urea treated soils in a lodge pole pine forest in Alberta, Canada were kindly provided by Dr. Akira Suzuki of Chiba University, Chiba, Japan and the University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, Alberta, Canada. Stock cultures of fungi were grown periodically on potato dextrose agar (PDA; Difco, Detroit, Michigan) at 25°C for 7 to 14 days, depending on the strains, and stored at 4°C.

5.2.2. Chemicals

Glucose (GO) assay kits, 96% veratryl alcohol (Aldrich brand), and 98% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Dextrose, PDA, Bacto® peptone, malt extract and yeast extract manufactured by Difco were purchased from Fisher Scientific Canada (Edmonton, Alberta, Canada). Other chemicals were purchased either from Fisher Scientific Canada or from Sigma-Aldrich Canada Ltd. Ultrapure water produced by Elgastat Maxima Water Purification System (Elga Ltd., High Wycombe, Bucks, England) was used for all reagent preparation.

5.2.3. Enzyme and chemical assays

Peroxidase activity was determined by a colorimetric assay using phenol and 4-aminoantipyrene as described previously (Ikehata and Buchanan, 2002). The activities of ligninolytic enzymes including LiP, MnP and laccase were measured with methods described in Pickard *et al.* (1999) using veratryl alcohol, Mn^{2+} and ABTS as a substrate, respectively. One unit of these enzymes is defined as one micromole of substrate oxidised per minute at 25°C. Glucose concentration was determined using a glucose oxidase/peroxidase method according to the Sigma glucose (GO) assay kit technical bulletin.

5.2.4. Peroxidase production

Seed culture for liquid fermentation was pre-grown on PDA at 25°C for 7 to 14 days depending on the fungal strain. Approximately 0.1 cm^3 of surface mycelia with agar from the seed culture was inoculated into 250 mL Erlenmeyer flasks containing 100 mL of a liquid medium composed of 1% glucose (dextrose), 0.5% Bacto® peptone, 0.3% yeast extract and 0.3% malt extract (YM broth) and cultivated on a rotary shaker at 175 rpm and 25°C. The initial pH of the culture broth was 6.2 ± 0.1. After a given time, triplicated culture flasks were harvested, and the liquid culture was centrifuged to remove fungal biomass at 2 000 g for 30 minutes at 4°C. The biomass was washed with pure water, dried in an oven at 70°C overnight and weighted. Clear supernatant was analysed for enzyme activities, glucose concentration and pH.

5.3. Results and discussion

5.3.1. Screening of Coprinus *species*

Screening experiments for the growth and peroxidase production of *Coprinus* species were carried out for 10 days at 25°C. The results are summarised in Table 5.1. The culture medium supported the growth of the tested fungal strains to varying degrees. Apparent peroxidase production was observed in the cultures of *Coprinus* spp. UAMH 10065, UAMH 10066, UAMH 10067 and 074. Minor peroxidase activity was detected in the cultures of *C. macrocephalus* NBRC 30117 and *C. echinosporus* NBRC 30630. No detectable peroxidase activity was found in the rest of the cultures. Laccase activity was found in some cultures of the tested fungi, including *C. macrocephalus* NBRC 30117, *C. radiatus* NBRC 30118, *C. phlyctidosporus* NBRC 30478, and *Coprinus* spp. UAMH 10065, UAMH 10066 and 074. The highest average laccase activity (0.1 U mL^{-1}) was found in the cultures of *C. radiatus* NBRC 30118. No activity was detected for other ligninolytic enzymes including LiP and MnP in any of the fungal cultures under the culture conditions used in this study.

Unlike the ligninolytic fungal peroxidases, LiP and MnP, which are commonly produced by a variety of white-rot fungi and whose physiological functions are known relatively well (Higuchi, 1990), non-ligninolytic peroxidases have been found only in the cultures of several *Coprinus* species and *A. ramosus*, and their physiological functions are largely unknown. In order to optimise the peroxidase production or to discover a better peroxidase producer for industrial or environmental applications, better understanding of the relationships between fungal ecology, physiology or taxonomy, and peroxidase production is highly desirable.

Table 5.1. Peroxidase production by *Coprinus* species grown in YM broth for 10 days.

Fungal strain	Growth	Peroxidase activity (U mL^{-1})
Coprinus echinosporus NBRC 30630	++	0.013
C. echinosporus NBRC 30631	++	ND
C. lagopides NBRC 30120	++	ND
C. macrocephalus NBRC 30117	+	0.013
C. neolagopus NBRC 30476	++	ND
C. neolagopus NBRC 32947	+++	ND
C. phlyctidosporus NBRC 30478	++	ND
C. phlyctidosporus NBRC 32946	+++	ND
C. radiatus NBRC 30118	+	ND
Coprinus sp. UAMH 10065	+	0.102
Coprinus sp. UAMH 10066	++	0.047
Coprinus sp. UAMH 10067	+++	10.4
Coprinus sp. 074	+	0.141

Note: Growth scoring: +, little growth (< 3 g dry biomass L^{-1}); ++, moderate growth (> 5 g dry biomass L^{-1}); +++, good growth (> 7 g dry biomass L^{-1}). Mean values of triplicates are shown for peroxidase activity. ND: not detected.

Our previous study demonstrated that several *Coprinus* species including *C. cinereus*, *C. lagopus* and a few unidentified *Coprinus* spp. were capable of producing extracellular non-ligninolytic peroxidase (Ikehata and Buchanan, 2002). According to the taxonomic classification, both of the identified *Coprinus* species, *C. cinereus* and *C. lagopus*, are close relatives and belong to the subsection *Lanatuli* of the genus *Coprinus* (Ulje and Noordeloos, 1999). In this study, other *Coprinus* species belonging to the subsection *Lanatuli*, including *C. lagopides*, *C. macrocephalus*, *C. neolagopus* and *C. radiatus*, were evaluated. However, except for *C. macrocephalus* NBRC 30117, these fungi failed to produce extracellular peroxidase under the same culture condition used in the previous study.

Our previous study also showed that the classical ecological classification could not explain the peroxidase production by certain *Coprinus* species (Ikehata and Buchanan, 2002). For example, although *C. cinereus* is a well-known coprophilous fungus, another coprophilous *Coprinus* species, *C. sterquilinus*, did not produce extracellular peroxidase. On the other hand, *C. lagopus* is not coprophilous but terrestrial. Other than the classical ecological classification, *C. cinereus* is a known member of a chemo-ecological group of fungi, "ammonia fungi", which form their fruiting bodies on the forest floor after decomposition of dead animal bodies or excrement, or after application of urea or any organic nitrogen compound that releases ammonia during decomposition (Suzuki, 1992; Sagara, 1995). Since it was considered that other *Coprinus* species belonging to this chemo-ecological group of fungi might produce extracellular peroxidase, unidentified *Coprinus* species that had been isolated from urea treated soil recently (*Coprinus* spp. UAMH 10065, UAMH 10066, UAMH 10067, and 074) as well as identified *Coprinus* species belonging to the ammonia fungi (*C. echinosporus*, *C. phlyctidosporus*, *C. neolagopus*), which had not been studied for peroxidase production, were evaluated in this study. Although the unidentified *Coprinus* species, especially *Coprinus* sp. UAMH 10067, clearly showed their peroxidase production ability, most of the identified strains tested, except for *C. echinosporus* NBRC 30630, failed to produce peroxidase under the culture condition used in this study. Consequently, the association between peroxidase production and ecological or taxonomic factors of *Coprinus* species is still unclear. It should be noted that the fungi failed to produce peroxidase in this study might produce it under different culture conditions, such as pH, nutrient composition and temperature. More studies, probably with genetic approaches, will be needed to clarify this issue.

Nevertheless, because some of the *Coprinus* species isolated from urea treated soil produced extracellular peroxidase, it can be suggested that the well-established method to isolate ammonia fungi (Sagara, 1995) may be used as an efficient method to isolate potential peroxidase producing *Coprinus* species from the field.

5.3.2. Production of peroxidase by Coprinus *sp. UAMH 10067*

Since *Coprinus* sp. UAMH 10067 produced a large amount of peroxidase, which was comparable to that produced by *C. cinereus* investigated previously (Ikehata and Buchanan, 2002), the time course of the growth and peroxidase production of this fungus was also studied. Figure 5.1 shows the growth and peroxidase production of *Coprinus* sp. UAMH 10067 for a 12-day period. Peroxidase activity was detected on day 5 and increased rapidly thereafter. The average peroxidase activity stayed nearly constant around 14 U mL^{-1} from day 8 until day 11 and started declining on day 12.

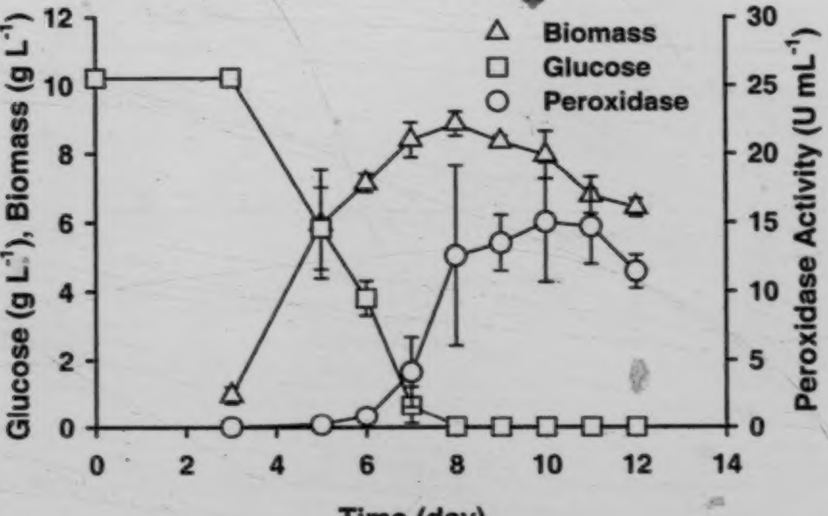


Figure 5.1. Production of peroxidase by *Coprinus* sp. UAMH 10067 in YM broth at 25°C. Mean of triplicates and standard deviation are shown.

The observed peak of peroxidase activity coincided with the exhaustion of medium glucose (Figure 5.1), which was also observed in the cultures of *C. cinereus* (Ikehata and Buchanan, 2002). Although the maximum average activity of peroxidase in the cultures of *Coprinus* sp. UAMH 10067 (15 U mL^{-1}) was less than that of CIP in *C. cinereus* UAMH 4103 cultures (28 U mL^{-1}) cultivated under the same condition, the peroxidase produced by *Coprinus* sp. UAMH 10067 appeared to be more stable than CIP, whose activity declined rapidly after it reached the peak in prolonged cultivation.

Higher stability of enzymes is generally preferable, and especially beneficial for their application to industrial wastewater treatment because the temperature of industrial wastewater tends to be higher than room temperature, at which enzyme inactivation may be accelerated. Preliminary stability test at room temperature (25°C) and an elevated temperature (50°C) on the crude enzymes also showed that the peroxidase from *Coprinus* sp. UAMH 10067 was likely more thermally stable than that from *C. cinereus* UAMH 4103 (Figure 5.2). Purification and further characterisation of the peroxidases from *C. cinereus* UAMH 4103 and *Coprinus* sp. UAMH 10067 is currently underway.

To date, the only *Coprinus* peroxidases characterised are CIP from *C. cinereus* IFO 30114 (NBRC 30114), IFO 30116 (NBRC 30116) and IFO 8371 (NBRC 8371) and *C. macrorhizus* peroxidase from an unspecified strain (Morita *et al.*, 1988; Kjalke *et al.*, 1992; Baunsgaard *et al.*, 1993). These studies concluded that CIP was identical in terms of the molecular weight, immunochemical identity and amino acid sequence to ARP and the peroxidase from *C. macrorhizus*, which used to be considered as a distinct species but now is regarded as *C. cinereus* (Orton and Watling, 1979). In addition, *A. ramosus*, the fungal source of ARP, is a deuteromycete having very similar taxonomic characteristics to the genus *Coprinus* (Sawai-Hatanaka *et al.*, 1995), although no definite evidence (*i.e.*, the complete lifecycle of this fungus) has yet been shown. The results of this study, however, suggest that not all *Coprinus* peroxidases have the same characteristics, and there are possibilities of discovering *Coprinus* strains that are capable of producing peroxidase with unique properties.

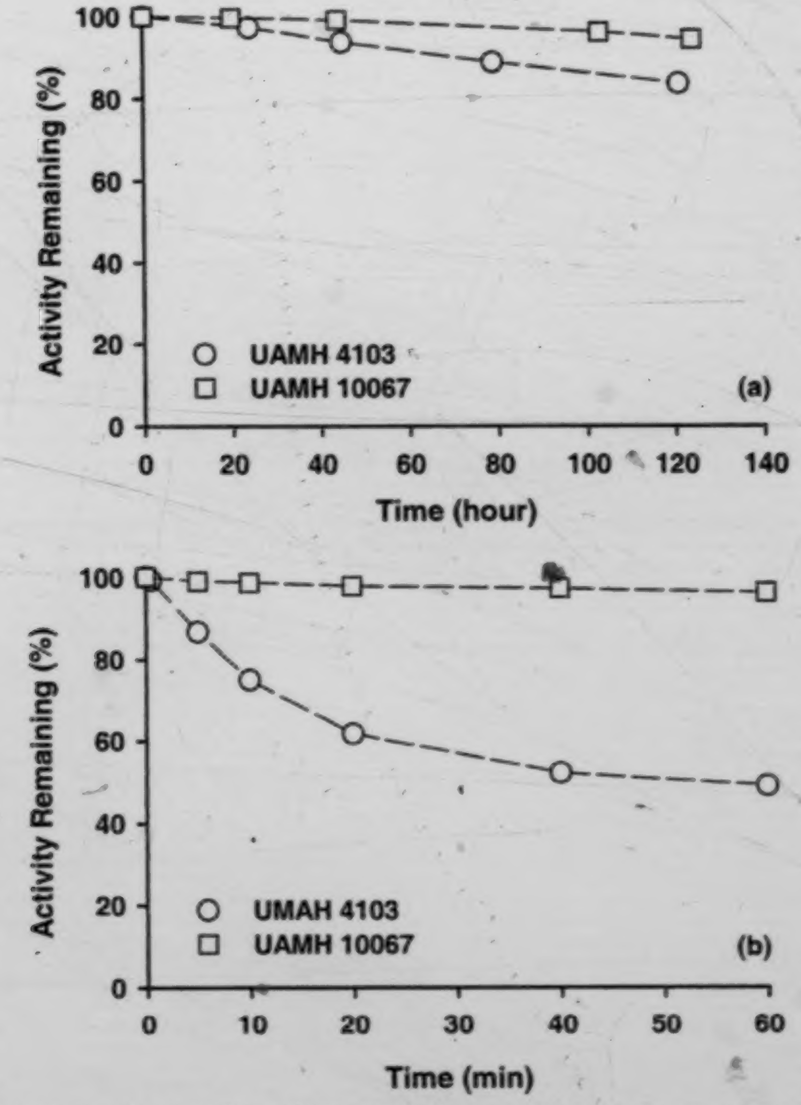


Figure 5.2. Comparison of the stability of crude peroxidases from *Coprinus cinereus* UAMH 4103 and *Coprinus* sp. UAMH 10067 at (a) 25°C and (b) 50°C in 0.1 M phosphate buffer at pH 7.0. Initial peroxidase activity was 1.3 U mL^{-1}. Average values of duplicated test results are shown.

References

Akimoto, K., Sinmen, Y., Sumida, M., Asami, S., Amachi, T., Yoshizumi, H., Saeki, Y., Shimizu, S. and Yamada, H. (1990). Luminol chemiluminescence reaction catalyzed by a microbial peroxidase. *Analytical Biochemistry* **189**: 182-185.

Al-Kassim, L., Taylor, K. E., Nicell, J. A., Bewtra, J. K. and Biswas, N. (1994b). Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxide from *Coprinus macrorhizus* in batch reactors. *Journal of Chemical Technology and Biotechnology* **61**: 179-182.

Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W. and Fu, P. C. (1974). Enzymatic determination of total serum cholesterol. *Clinical Chemistry* **20**(4): 470-475.

Baunsgaard, L., Dalboge, H., Houen, G., Rasmussen, E. M. and Welinder, K. G. (1993). Amino acid sequence of *Coprinus macrorhizus* peroxidase and cDNA sequence encoding *Coprinus cinereus* peroxidase. A new family of fungal peroxidases. *European Journal of Biochemistry* **213**: 605-611.

Buchanan, I. D. and Han, Y. S. (2000). Assessment of the potential of *Arthromyces ramosus* peroxidase to remove phenol from industrial wastewaters. *Environmental Technology* **21**: 545-552.

Dordick, J. S. (1992). Enzymatic and chemoenzymatic approaches to polymer synthesis. *Trends in Biotechnology* **10**: 287-293.

Higuchi, T. (1990). Lignin biochemistry: Biosynthesis and biodegradation. *Wood Science and Technology* **24**: 23-63.

Ikehata, K. and Buchanan, I. D. (2002). Screening of *Coprinus* species for the production of extracellular peroxidase and evaluation of the enzyme for the treatment of aqueous phenol. *Environmental Technology* **23**(12): 1355-1368.

Kim, B. B., Pisarev, V. V. and Egorov, A. M. (1991). A comparative study of peroxidases from horse radish and *Arthromyces ramosus* as labels in luminol-mediated chemiluminescent assays. *Analytical Biochemistry* **199**: 1-6.

Kjalke, M., Andersen, M. B., Schneider, P., Christensen, B., Schulein, M. and Welinder, K. G. (1992). Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. *Biochimica et Biophysica Acta* **1120**: 248-256.

Klibanov, A. M., Alberti, B. N., Morris, E. D. and Felshin, L. M. (1980). Enzymatic removal of toxic phenols and anilines from waste waters. *Journal of Applied Biochemistry* **2**: 414-421.

Masuda, M., Sakurai, A. and Sakakibara, M. (2001). Effect of reaction conditions on phenol removal by polymerization and precipitation using *Coprinus cinereus* peroxidase. *Enzyme and Microbial Technology* **28**: 295-300.

Morita, Y., Yamashita, H., Mikami, B., Iwamoto, H., Aibara, S., Terada, M. and Minami, J. (1988). Purification, crystallization, and characterization of peroxidase from *Coprinus cinereus*. *Journal of Biochemistry* **103**(4): 693-699.

Nicell, J. A., Bewtra, J. K., Biswas, N., Pierre, C. C. S. and Taylor, K. E. (1993). Enzyme catalyzed polymerization and precipitation of aromatic compounds from aqueous solution. *Canadian Journal of Civil Engineering* **20**: 725-735.

Orton, P. D. and Watling, R. (1979). Coprinaceae Part 1: Coprinus. in *British Fungus Flora Agarics and Boleti*. Henderson, D. M., Orton, P. D. and Watling, R., Editors. Royal Botanic Garden, Edinburgh, Her Majesty's Stationery Office:

7-125.

Pickard, M. A., Vandertol, H., Roman, R. and Vazquez-Duhalt, R. (1999). High production of ligninolytic enzymes from white rot fungi in cereal bran liquid medium. *Canadian Journal of Microbiology* **45**: 627-631.

Sagara, N. (1995). Association of ectomycorrhizal fungi with decomposed animal wastes in forest habitats: a cleaning symbiosis? *Canadian Journal of Botany* **73**(Suppl. 1): S1423-S1433.

Sawai-Hatanaka, H., Ashikari, T., Tanaka, Y., Asada, Y., Nakayama, T., Minakata, H., Kunishima, N., Fukuyama, K., Yamada, H., Shibano, Y. and Amachi, T. (1995). Cloning, sequencing, and heterologous expression of a gene coding for *Arthromyces ramosus* peroxidase. *Bioscience, Biotechnology, and Biochemistry* **59**(7): 1221-1228.

Shinmen, Y., Asami, S., Amachi, T., Shimizu, S. and Yamada, H. (1986). Crystallization and characterization of an extracellular fungal peroxidase. *Agricultural and Biological Chemistry* **50**(1): 247-249.

Smith, K., Silvernail, N. J., Rodgers, K. R., Elgren, T. E., Castro, M. and Parker, R. M. (2002). Sol-gel encapsulated horseradish peroxidase: A catalytic material for peroxidation. *Journal of the American Chemical Society* **124**(16): 4247-4252.

Suzuki, A. (1992). Physiological characteristics of the ammonia fungi and their role in bioscience. *Proceedings of the Asian Mycological Symposium*, Seoul, Korea, Korean Society of Mycology: 238-247.

Ulje, C. B. and Noordeloos, M. E. (1999). Studies in Coprinus V - Coprinus section Coprinus Revision of subsection Lanatuli Sing. *Persoonia* **17**(2): 165-199.

Villalobos, D. A. and Buchanan, I. D. (2002). Removal of aqueous phenol by

Arthromyces ramosus peroxidase. *Journal of Environmental Engineering and Science* **1**: 65-73.

Chapter 6. Purification, Characterisation and Evaluation of Extracellular Peroxidase from Two *Coprinus* Species for Aqueous Phenol Treatment*

6.1. Introduction

Phenolic compounds are one of the major classes of organic pollutants generated through various industrial activities. For example, more than 97,000 tonnes of phenolic wastes, including phenol, 2-phenylphenol, 4,4’-isopropylidenediphenol (bisphenol A), catechol, chlorophenols, nitrophenols and cresols, were generated by the industries in the United States in 2000 (US EPA, 2002). Of the phenolic wastes generated in 2000, approximately 6,800 tonnes were released to the environment through air emission, surface water discharge, land disposal and underground injection (US EPA, 2002). Although the toxicity and environmental impacts of phenolic compounds vary depending on the numbers, types and positions of substituted groups on the aromatic ring(s), these chemicals are considered to be toxic to various organisms including humans (National Toxicology Program, 2003). In addition to the acute toxicities, some phenolic pollutants such as bisphenol A and alkylphenols possess potential endocrine disrupting activities even at very low concentrations (Servos, 1999; Singleton and Khan, 2003). Some substituted phenols, such as chlorophenols and alkylphenols, are also highly biorefractory, so that conventional biological processes cannot effectively remove these compounds (Karam and Nicell, 1997; Bennie, 1999). Thus, the development of more effective treatment processes is desired for the removal of these phenolic pollutants from industrial waste streams and the environment.

---

* A version of this chapter (by Ikehata, K., Buchanan, I.D., Pickard, M.A. and Smith, D.W.) has been submitted to *Bioresource Technology* in August 2003.

Among the novel treatment options, an enzymatic process using a peroxidase, such as horseradish peroxidase (HRP), and hydrogen peroxide has proven very effective in removing a variety of phenolic pollutants from aqueous solutions with high efficiency and selectivity (Klibanov *et al.*, 1980; Nicell *et al.*, 1993; Wu *et al.*, 1997). Peroxidase (donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7) catalyses the dehydrogenation of varieties of phenols and anilines in the presence of hydrogen peroxide. As shown in Figure 6.1, hydrogen peroxide first oxidises peroxidase to a catalytically active form called Compound I that is capable of oxidising a phenolic substrate to a phenoxy radical by the one-electron oxidation. Compound I is thereby reduced to Compound II, a second catalytically active form, that can oxidise a second phenolic substrate to generate another phenoxy radical and return the enzyme to its resting state. Compound III is the relatively inactive form of enzyme, which is slowly decomposed to resting enzyme or returns to Compound I through another one-electron oxidation of phenol. The phenoxy radicals generated through the catalytic reactions spontaneously polymerise to form water insoluble phenolic polymers that can be removed by gravity separation or filtration (Klibanov *et al.*, 1980).

Although a great deal of research has been carried out to demonstrate the effectiveness of HRP-catalysed removal of phenolic pollutants either from synthetic aqueous mixtures (Klibanov *et al.*, 1980; Nicell *et al.*, 1993; Wu *et al.*, 1997; Zhang and Nicell, 2000) or from real wastewaters (Klibanov *et al.*, 1983; Nakamoto and Machida, 1992; Cooper and Nicell, 1996; Wagner and Nicell, 2001b; Wagner and Nicell, 2001a), the implementation of this process on a large-scale is still hampered primary by the high

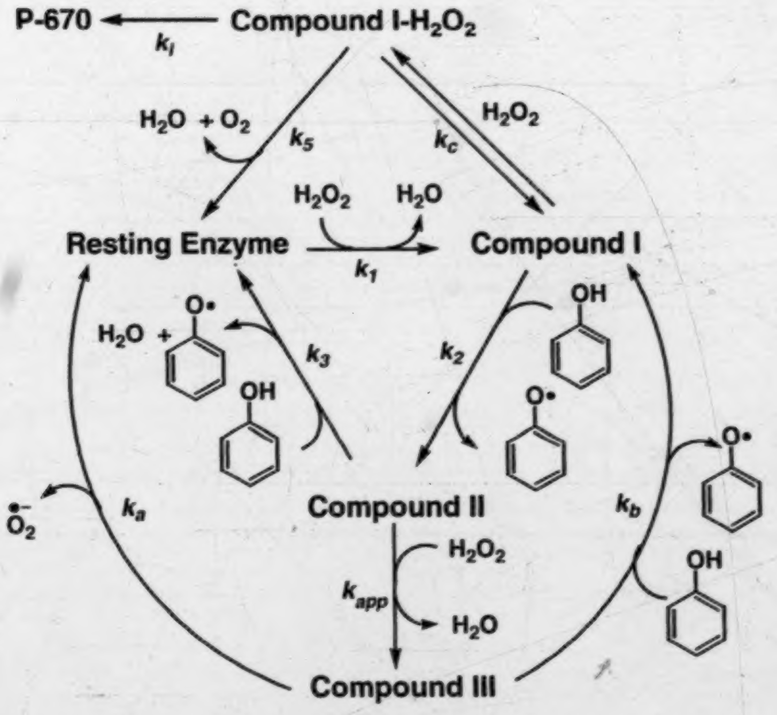

P-670
k_i
Compound I-H_2O_2
$H_2O + O_2$
k_5
k_c
H_2O_2
H_2O_2
H_2O
Resting Enzyme
k_1
Compound I
O•
H_2O +
OH
k_3
k_2
OH
O•
$\overset{\bullet -}{O_2}$
k_a
Compound II
k_b
O•
H_2O_2
OH
k_{app}
H_2O
Compound III

Figure 6.1. Catalytic cycle of peroxidase. P-670 is a permanently inactivated form of enzyme called verodohaemoprotein.

cost associated with the production of the enzyme. Several other plant and fungal peroxidases, including soybean peroxidase (SBP; Caza *et al.*, 1999; Flock *et al.*, 1999; Wright and Nicell, 1999; Kinsley and Nicell, 2000), *Arthromyces ramosus* peroxidase (ARP; Buchanan and Han, 2000; Ibrahim *et al.*, 2001; Villalobos and Buchanan, 2002) and *Coprinus cinereus* (also known as *C. macrorhizus*) peroxidase (CIP; Al-Kassim *et al.*, 1994a; 1994b; Kauffmann *et al.*, 1999; Masuda *et al.*, 2001a; Ikehata and Buchanan, 2002; Ikehata *et al.*, 2003a) have been investigated as potential alternatives to HRP. The latter two fungal enzymes are particularly promising for large-scale production because the parent fungi, *A. ramosus* and *C. cinereus*, grow fairly quickly and excrete extracellular peroxidase in large quantity to the liquid medium (Shinmen *et al.*, 1986;

Morita *et al.*, 1988; Yao *et al.*, 1995; Ikehata and Buchanan, 2002; Sakurai *et al.*, 2002). In addition to these two fungi, production of extracellular peroxidase by other *Coprinus* species such as *C. lagopus* was also demonstrated recently (Ikehata and Buchanan, 2002; Ikehata *et al.*, 2003b).

In our previous fungal survey, *C. cinereus* UAMH 4103 was found to be one of the best peroxidase producing *Coprinus* species (Ikehata and Buchanan, 2002). It was also found that the crude peroxidase produced by a newly isolated *Coprinus* sp. UAMH 10067 appeared to be more stable at high temperature (50°C) than the crude enzyme produced by *C. cinereus* (Ikehata *et al.*, 2003b). High thermal stability of the enzyme is particularly beneficial for its application to industrial wastewater treatment, because the temperature of industrial wastewaters tend to be higher than room temperature, and may accelerate enzyme inactivation. In this study, the extracellular peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 were purified, characterised and evaluated for the phenolic wastewater treatment. Some catalytic properties, stability and aqueous phenol treatment efficiency of these enzymes in the form of crude filtrate of fungal culture fluid and in the purified form were compared in order to elucidate the effect of purification on the *Coprinus* peroxidase-catalysed treatment of phenolic wastewater. The results were also compared with published data for the previously studied plant peroxidases.

6.2. Materials and methods

6.2.1. Enzymes and chemicals

Peroxidases (EC 1.11.1.7) from horseradishes (HRP; RZ = 1.1) and from *A.*

ramosus (ARP; RZ = 2.5), poly(ethylene glycol) (PEG; average M.W. = 32 kDa, Fluka Brand), 99% 3,5-dichloro-2-hydroxybenzenesulphonic acid sodium salt (HDCBS; Aldrich brand) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). 4-Aminoantipyrine (4-AAP) was purchased from Fisher Scientific Canada (Edmonton, Alberta, Canada). Other chemicals were purchased either from Sigma-Aldrich Canada or Fisher Scientific Canada. Ultrapure water produced by Elgastat Maxima Water Purification System (Elga Ltd., High Wycombe, Bucks, England) was used for all reagent preparation.

6.2.2. Fungi and peroxidase production

Coprinus sp. UAMH 10067 and *C. cinereus* UAMH 4103 were obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, Alberta, Canada. Stock cultures of these fungi were grown periodically on potato dextrose agar (PDA; Difco brand, Becton Dickinson, Sparks, Maryland) at 25°C for 7 to 10 days and stored at 4°C. Seed cultures for liquid fermentation were grown on PDA plates at 25°C for 7 to 10 days prior to the fermentation experiments.

Inocula were prepared by homogenising approximately 20 cm^2 of surface mycelium from a seed culture plate in 100 mL of liquid medium in a Sorvall Omnimixer (Sorvall, Norwalk, Connecticut). Four milliliters of homogenised mycelium were inoculated in 500 mL Erlenmeyer flasks containing 150 mL of a liquid medium containing 1% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract (all medium ingredients were Difco brand) and cultivated on a rotary shaker at 200 rpm and 27 ± 1°C for 7 to 9 days or until the medium glucose was consumed completely, and the peroxidase activity had nearly reached its maximum for the given growth conditions

(Ikehata and Buchanan, 2002; Ikehata *et al.*, 2003b). The liquid culture was subsequently filtered by filter paper to remove the larger fraction of the fungal biomass, and the filtrate was centrifuged at approx. 5 000 *g* at 4°C for 20 minutes to remove finer particles. This culture filtrate was used as a stock of crude enzyme. Normally, the peroxidase activity in the filtrate was 10 to 14 U mL^{-1} for *Coprinus* sp. UAMH 10067 and 20 to 28 U mL^{-1} for *C. cinereus* UAMH 4103 based on a colourimetric assay with phenol and 4-AAP described below.

6.2.3. Peroxidase activity assay

Peroxidase activity was determined by a colourimetric assay using phenol and 4-AAP described in Wright and Nicell (1999). The 1 mL assay mixture was composed of 500 μl of 20 mM phenol, 250 μl of 9.6 mM 4-AAP, 100 μl of 2 mM hydrogen peroxide, 0 to 130 μl of 0.1 M sodium phosphate buffer at pH 7.4 and 20 to 150 μl of enzyme solution. All reagents were prepared in 0.1 M sodium phosphate buffer adjusted to pH 7.4. Immediately after the addition of sample, the formation of quinoneimine dye was monitored for 30 seconds using a spectrophotometer at a wavelength of 510 nm at 25°C. One unit (U) of peroxidase activity is defined as one micromole of hydrogen peroxide consumed in one minute at pH 7.4 and 25°C using a conversion factor of 7100 M^{-1} cm^{-1} based on hydrogen peroxide.

6.2.4. Peroxidase purification

Several batches of the *Coprinus* species culture filtrate, prepared as above, were pooled and frozen at –20°C. When thawed, much of the extracellular polysaccharide and some pigment produced by fungus formed a floc that was removed by centrifugation at

5 000 *g* for 20 minutes. The supernatant was concentrated by ultrafiltration using a Prep/Scale-TFF cartridge (molecular weight cut off = 10 kDa; Millipore Corp., Bedford, Massachusetts) for initial concentration, and a PM-10 membrane (Amicon, Lexington, Massachusetts) for further concentration of smaller volume of the enzyme solution. The concentrated enzyme solution was dialysed extensively against 10 mM potassium phosphate buffer at pH 7 (simply referred as "buffer" in the following) to remove residuals of medium ingredients and other smaller compounds, and subsequently applied to a DEAE-cellulose anion-exchange column (Whatman DE-52, Maidstone, England). The column was washed with 2 empty bed volumes of buffer. A shallow linear gradient from 0 to 300 mM KCl in buffer was applied to the column to elute peroxidase. The peroxidase was eluted at around 80 mM KCl. For the peroxidase from *Coprinus* sp. UAMH 10067, the peak fractions were pooled, concentrated and dialysed by ultrafiltration, applied to the DEAE-cellulose column for a second time, and eluted with the same gradient of KCl described above. The peak fractions from the DEAE-cellulose column were pooled, concentrated by ultrafiltration, and applied to a size exclusion column (Sephadex G-100, Sigma) and eluted with 100 mM potassium phosphate buffer at pH 7. The highest peak fraction from the Sephadex column was selected, concentrated, dialysed and applied to an anion exchange fast protein liquid chromatography (FPLC) column (MonoQ HR5/5, Pharmacia). The same gradient of KCl as used with the DEAE-cellulose column was used to elute enzyme from the FPLC column. Enzyme activity and Reinheitzahl (RZ) ratio (A_{405}/A_{280}) of the enzyme solution were recorded at each purification step. Protein concentrations in the purified enzyme solution were determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, California) and bovine serum albumin as a standard.

6.2.5. Peroxidase characterisation

The molecular mass of *Coprinus* peroxidase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide gel) with marker proteins (Sigma), as well as by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) using sinapinic acid as the matrix and bovine serum albumin for external calibration. The MALDI-TOF MS analysis was carried out with a Voyager DE-Pro (Applied Biosystems) at the Institute for Biomolecular Design, University of Alberta, Canada. The amino acid composition of *Coprinus* peroxidase was determined using a Beckman System 6300 Amino Acid Analyzer at the Alberta Peptide Institute, University of Alberta.

6.2.6. **Coprinus** *peroxidase activity as a function of pH and hydrogen peroxide concentration*

The effect of assay pH and hydrogen peroxide concentration on the catalytic activity of *Coprinus* peroxidase was investigated by varying the pH and hydrogen peroxide concentration in the activity assay mixture. The following buffers were used: citrate buffer for pH from 3 to 5; phosphate buffer for pH from 6 to 8; and carbonate buffer for pH from 9 to 10. The rate of colour generation was monitored at the wavelength of 510 nm and converted to an apparent activity. This was later expressed as a relative activity, with respect to that measured under the standard assay conditions.

6.2.7. Stability of **Coprinus** *peroxidase*

The stability of *Coprinus* peroxidase was investigated by monitoring the change in peroxidase activity after incubation in various pH buffers or at different temperatures. The same buffer salts used in pH-activity tests described above were used in pH stability experiments. The buffered enzyme solution was properly diluted with pH 7.4 phosphate buffer prior to the peroxidase assay, so that the assay pH was not affected. In cases where the enzyme solution was incubated at an elevated temperature, an aliquot of sample withdrawn from the incubated enzyme solution was quickly cooled in an ice water bath to halt the inactivation. Once cooled, the sample was removed from the water bath, allowed to warm to 25°C and the peroxidase activity in the sample was measured according to the standard activity assay at 25°C and pH 7.4.

6.2.8. Batch treatment of aqueous phenol with **Coprinus** *peroxidase*

All batch treatment experiments were carried out in 30 mL borosilicate vials containing 10 mL reaction mixture that was composed of 1.1 mM phenol and 1.5 mM hydrogen peroxide at 25°C. The reaction mixture also contained 0.1 M of buffer salts, which were the same buffer species as those used in the pH-activity tests. A concentrated PEG solution was also added in some vials to a final concentration of 200 mg L^{-1} before the reaction was initiated. In order to initiate the reaction, an aliquot of peroxidase solution was added to the stirred reaction mixture. After 3 hours, the reaction mixture was centrifuged at 2 000 *g* for 30 minutes to removed suspended materials. Clear supernatant was subjected to the analyses for residual peroxidase activity, and concentrations of phenol and hydrogen peroxide. Two controls were run concurrently with each set of treatments: a reaction mixture without hydrogen peroxide, and one with hydrogen peroxide but without enzyme. No change in phenol concentration was observed in any of these controls. Each treatment was duplicated and the mean values are presented.

6.2.9. Chemical assays

Concentrations of phenol and hydrogen peroxide were determined by colourimetric assays described previously (Ikehata and Buchanan, 2002), using 4-AAP and potassium ferricyanide and using HDCBS, 4-AAP and HRP, respectively.

6.3. Results

6.3.1. Purification and characterisation of Coprinus *peroxidase*

The purification data for the peroxidase from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 are presented in Tables 6.1 and 6.2. After FPLC purification, the RZ value and specific activity of these enzyme preparations were 2.27 and 372 U mg^{-1}, and 2.50 and 440 U mg^{-1}, respectively. Although two fractions of the former peroxidase from FPLC are shown in Table 6.1, only the purest fraction (#1) was characterised further. The purified *Coprinus* peroxidases and a commercial preparation of ARP, which is known to be identical to *C. cinereus* peroxidase (CIP) (Kjalke *et al.*, 1992), were analysed by SDS-PAGE. There are a group of three to four major bands around 36 kDa and faint bands around 30 kDa in the peroxidase from *C. cinereus* UAMH 4103, while only the major bands around 36 kDa are evident in the peroxidase from *Coprinus* sp. UAMH 10067 in the SDS-polyacrylamide gel (Figure 6.2). The bands around 30 kDa were more apparent in the commercial preparation of ARP. The MALDI-TOF MS analyses of the purified *Coprinus* peroxidase preparations revealed that there were multiple forms of peroxidases in the samples: at least five in the purified peroxidase from *C. cinereus* UAMH 4103 (36.15, 36.31, 36.47, 36.63 and 36.84 kDa) and two in that from *Coprinus* sp. UAMH 10067 (36.16 and 36.32 kDa). These multiple forms were thought to be active isoenzymes, because polyacrylamide electrophoresis under un-denaturing condition also gave multiple bands as the gel was stained with *o*-dianisidine and hydrogen peroxide (data not shown). No signal corresponding to 30 kDa, which was observed by SDS-PAGE, was detected in the MALDI-TOF mass spectrum for either enzyme.

Table 6.1. Purification of peroxidase from *Coprinus* sp. UAMH 10067.

	Activity ($U\ mL^{-1}$)	Volume (mL)	Total Activity (U)	Yield (%)	RZ
Culture supernatant	8	8950	69505	100.0	NP
Ultrafiltration	267	198	52932	76.2	NP
1st DEAE pool	246	147	36168	52.0	0.46
2nd DEAE pool	391	78	30515	43.9	1.14
Sephadex peak fraction	564	24	13534	19.5	1.76
FPLC peak fraction #1	184	1	184	0.3	2.27
FPLC peak fraction #2	192	1	192	0.3	2.06

Note: NP = no peak around 400 nm.

Table 6.2. Purification of peroxidase from *C. cinereus* UAMH 4103.

	Activity ($U\ mL^{-1}$)	Volume (mL)	Total Activity (U)	Yield (%)	RZ
Culture supernatant	23	5150	115980	100.0	NP
Ultrafiltration	1004	83	83332	71.9	NP
DEAE pool	415	113	46895	40.4	1.01
Sephadex peak fraction	983	9	9240	8.0	2.37
FPLC peak fraction	280	1	280	0.2	2.50

Note: NP = no peak around 400 nm.

Results of the amino acid composition analyses for purified *Coprinus* peroxidases are presented in Table 6.3. It was assumed that the total numbers of amino

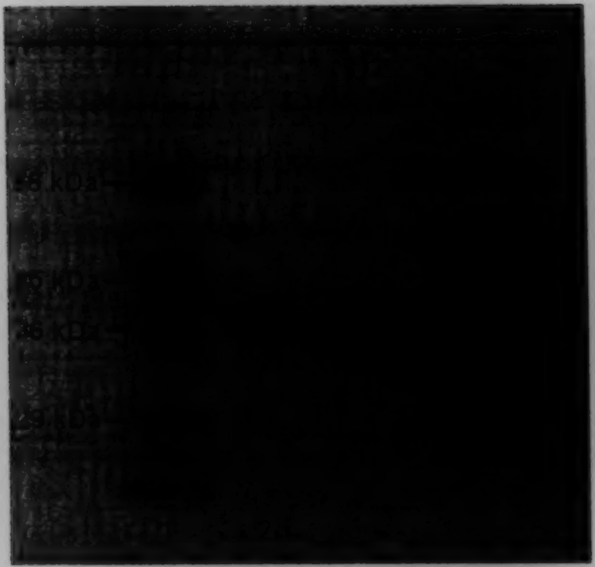


Figure 6.2. SDS-PAGE of purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 (lane 1: MW marker, lane 2: UAMH 10067 fraction #1, lane 3: UAMH 10067 fraction #2, lane 4: UAMH 4103, lane 5: ARP from Sigma).

acid residues and the numbers of cysteine and tryptophan residues, which were decomposed during acid hydrolysis, of these proteins were the same as those of previously studied CIP (343 residues in total, 8 cysteine residues and 2 tryptophan residues) (Kjalke *et al.*, 1992; Baunsgaard *et al.*, 1993). Published amino acid compositions of CIP and ARP (Kjalke *et al.*, 1992), as well as the amino acid compositions of CIP and ARP derived from the deduced amino acid sequences from the nucleotide sequences of the peroxidase cDNA (Baunsgaard *et al.*, 1993; Sawai-Hatanaka *et al.*, 1995), are also shown in Table 6.3. With some minor variations, the compositions of two *Coprinus* peroxidases studied here are very similar to the published values. Although N-terminal sequencing for the current *Coprinus* peroxidase preparations was also attempted using automated Edman degradation (performed at the Alberta Peptide Institute), it was unsuccessful probably due to the N-terminal blocking by pyroglutamic acid (Sawai-Hatanaka *et al.*, 1995).

Table 6.3. Amino acid compositions of peroxidase from *Coprinus* sp. UAMH 10067, *C. cinereus* UAMH 4103, and published values for CIP and ARP.

Residue	*Coprinus* sp. UAMH 10067	*C. cinereus* UAMH 4103	CIP[†]	ARP[†]	CIP, ARP[‡]
Asp + Asn	33.8	34.9	34.5	34.0	33
Thr	18.1	19.1	19.3	19.7	20
Ser	34.2	34.8	36.2	38.0	38
Glu + Gln	31.4	31.3	29.4	28.9	28
Gly	37.3	37.7	37.9	38.4	37(38)[¶]
Ala	33.1	33.3	33.6	33.0	33
Cys	8.0[*]	8.0[*]	7.5	7.0	8
Val	18.2	19.2	19.8	18.9	19
Met	5.3	5.9	6.5	6.2	7
Ile	14.5	14.9	14.6	15.5	17
Leu	28.5	29.3	28.6	28.4	28
Tyr	4.5	3.4	3.1	3.6	3
Phe	18.1	18.4	17.8	17.4	18
His	5.1	5.0	4.2	4.4	4
Lys	4.9	4.7	4.1	4.3	4
Trp	2.0[*]	2.0[*]	1.9	2	2
Arg	15.8	15.7	15.3	15.4	15
Pro	30.2	25.2	28.7	27.7	29
Total	343[*]	343[*]	343	343	343(344)[¶]

Note:

[*] Total number of residues and the numbers of cysteine and trptophan residues were assumed to be the same as the reported values (343, 8 and 2, respectively).

[†] CIP from *C. cinereus* IFO 30114 and ARP from Sigma (Kjalke et al., 1992).

[‡] From the deduced amino acid sequences from the nucleotide sequences of CIP cDNA (Baunsgaard et al., 1993) and ARP cDNA (Sawai-Hatanaka et al., 1995).

[¶] A difference in the numbers of glycine residues (37 residues in CIP and 38 residues in ARP) was reported.

6.3.2. **Coprinus** ***peroxidase activity as a function of pH***

Effect of pH on the activity of crude and purified *Coprinus* peroxidase was investigated by modifying pH in the standard peroxidase assay described in Materials and Methods. The results are shown in Figure 6.3. There is no marked difference in the shape of the relative activity versus pH plots between the peroxidase from *Coprinus* sp. UAMH 10067 and that from *C. cinereus* UAMH 4103. The highest activity was observed around pH 6 for both enzymes, although theses enzymes were very active (more than 90% in relative activity) within the range of pH 6 to 8. Purified enzymes are less active from pH 7 to 8 than crude enzyme for both enzymes.

6.3.3. **Coprinus** ***peroxidase activity as a function of hydrogen peroxide concentration***

The effect of initial hydrogen peroxide concentration on the peroxidase activity of crude and purified peroxidase from two *Coprinus* species was investigated at pH 7.4 and 25°C with various enzyme concentrations. The results are presented in Figure 6.4, where peroxidase activity is expressed as activity relative to the activity measured under the standard assay condition ($[H_2O_2]_0 = 0.2$ mM).

The shapes of relative peroxidase activity versus initial hydrogen peroxide concentration curves are nearly identical for both crude and purified peroxidase from *Coprinus* sp. UAMH 10067 and from *C. cinereus* UAMH 4103. The maximum peroxidase activity was observed at the initial hydrogen peroxide concentration of about 0.2 mM in all cases. At lower hydrogen peroxide concentration, the catalytic cycle of peroxidase is limited by the formation of Compound I (see Figure 6.1). At higher hydrogen peroxide concentration, on the other hand, the excess hydrogen peroxide reacts with Compound II to form relatively inactive Compound III, which slowly returns to the

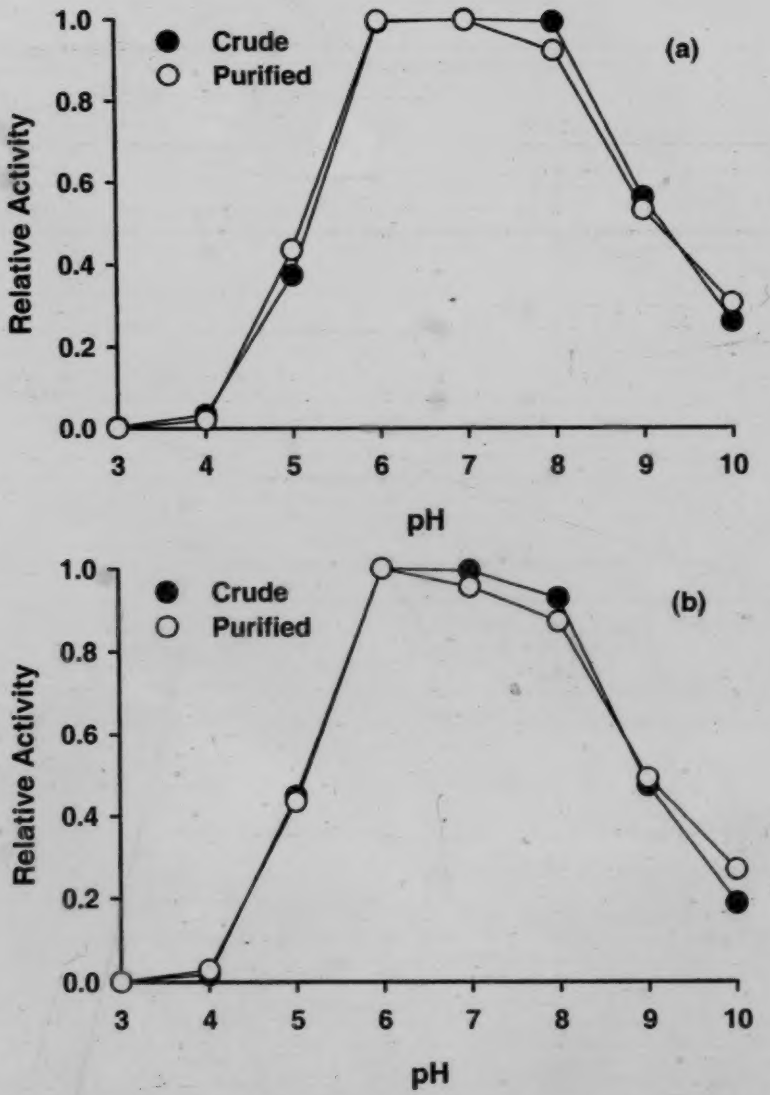


Figure 6.3. Effect of pH on the activity of peroxidases from (a) *Coprinus* sp. UAMH 10067 and (b) *C. cinereus* UAMH 4103. Average values of duplicated assays of two peroxidase solutions with different enzyme concentrations (approximately 0.015 U mL^{-1} and 0.030 U mL^{-1}) are shown.

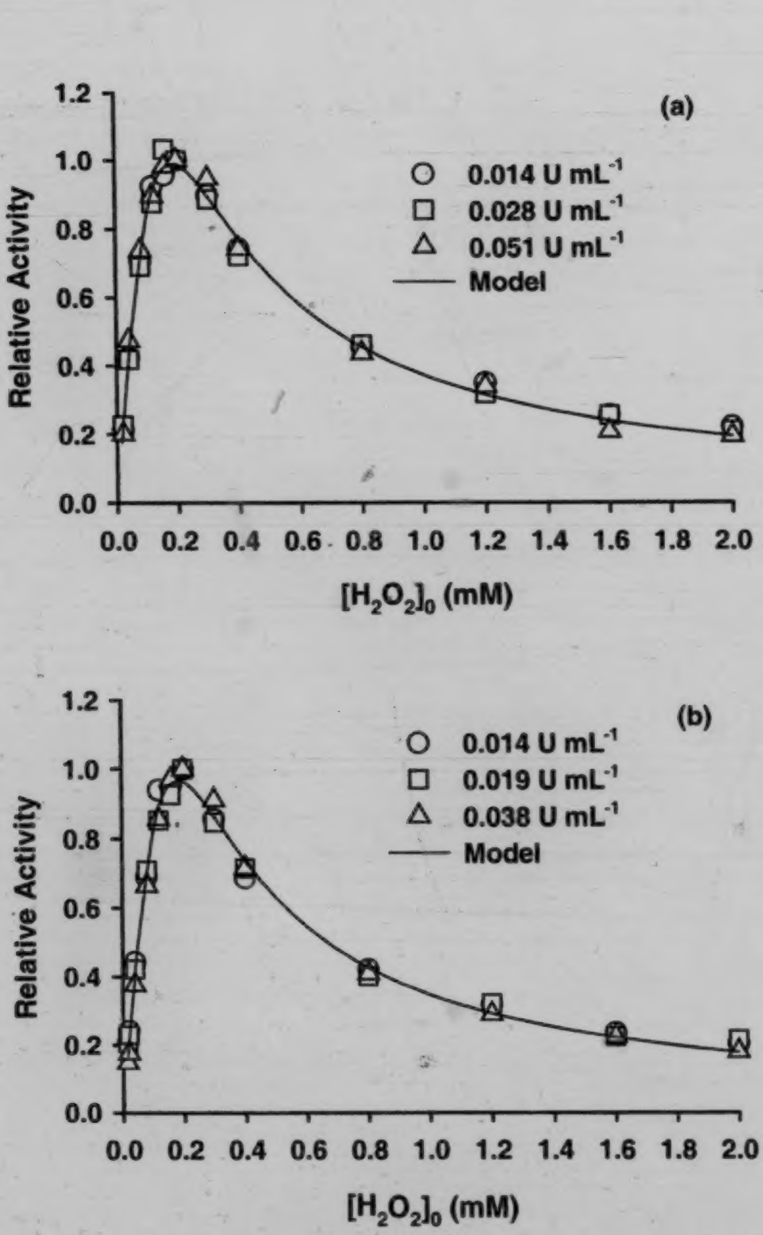


Figure 6.4. Effect of hydrogen peroxide concentration on the peroxidase activity of *Coprinus* peroxidase: (a) crude enzyme from *Coprinus* sp. UAMH 10067, (b) purified enzyme from *Coprinus* sp. UAMH 10067, (c) crude enzyme from *C. cinereus* UAMH 4103, and (d) purified enzyme from *C. cinereus* UAMH 4103. (See next page.)

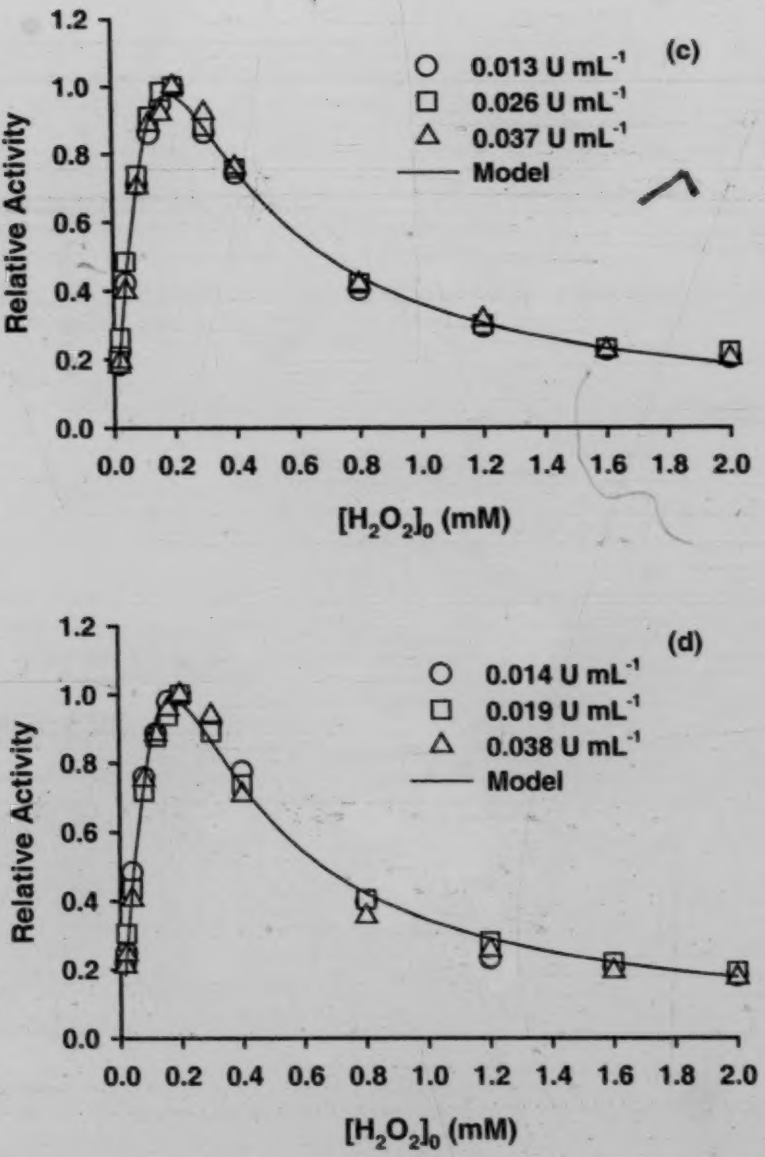


Figure 6.4. Effect of hydrogen peroxide concentration on the peroxidase activity of *Coprinus* peroxidase: (a) crude enzyme from *Coprinus* sp. UAMH 10067, (b) purified enzyme from *Coprinus* sp. UAMH 10067, (c) crude enzyme from *C. cinereus* UAMH 4103, and (d) purified enzyme from *C. cinereus* UAMH 4103 (continued).

central catalytic cycle shown in Figure 6.1. Since the reaction rate of the Compound III conversion is very slow compared with other reactions for peroxidase (Buchanan and Nicell, 1999), the Compound III formation results in the reduction in the hydrogen peroxide consumption rate at high hydrogen peroxide concentration.

Assuming steady-state hydrogen peroxide consumption during the peroxidase activity assay, some kinetic parameters for the peroxidase activity of two *Coprinus* peroxidases were estimated using the data presented in Figure 6.4 and a model developed by Nicell and Wright (1997). The peroxidase activity was converted to molar concentration of peroxidase using the proportionality constant, C_a, of 3.85×10^{-11} mol U^{-1}, which is based on the molar absorptivities of CIP at 405 nm (= 109 mM-1 cm-1; Andersen *et al.*, 1991). The estimated model parameters, α, β and γ, and the kinetic rate constants, k_1 and $(1/k_2 + 1/k_3)^{-1}$, derived from the estimated model parameters are presented in Table 6.4. The hydrogen peroxidase concentration at which maximum peroxidase activity occurs ($[H_2O_2]_{maxR}$) was also calculated using the model equations, and is also presented in Table 6.4.

All estimates for α, β and γ were statistically significant ($p < 0.05$) except for the α values for purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103. There is no statistical difference in the corresponding estimates of α, β and γ between any combinations of four samples of *Coprinus* peroxidase, including crude and purified peroxidase from *Coprinus* sp. UAMH 10067 and those from *C. cinereus* UAMH 4103 ($p > 0.05$, d.f. = 69 or 72). This implies that there is also no statistical difference between the corresponding kinetic rate constant or the $[H_2O_2]_{maxR}$ values calculated from α, β or γ. The estimated k_1 values for the two enzymes were approximately 2.5×10^{-6} mol^{-1} L s^{-1}, regardless of the enzyme purity. The $[H_2O_2]_{maxR}$ values were also nearly identical (approximately 0.18 mM) for all four samples of the peroxidase. The estimated $(1/k_2 + 1/k_3)^{-1}$ values varied from 3.6 × 10^5 (crude enzyme from UAMH 10067) to 1.7 × 10^6 mol^{-1} L s^{-1} (purified enzyme from UAMH 10067). However, this difference is not considered to be significant based on the statistical judgment shown above.

Table 6.4. Estimated kinetic model parameters for the peroxidase activity of the peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 at pH 7.4 and 25°C. Standard errors are presented for the estimates of α, β and γ.

		UAMH 10067		UAMH 4103	
Parameter	Unit	Crude	Purified	Crude	Purified
α	-	0.062 ± 0.032	0.014* ± 0.039	0.059 ± 0.030	0.036* ± 0.044
β	10^{-5} mol L^{-1}	8.85 ± 0.30	9.38 ± 0.36	8.87 ± 0.28	8.62 ± 0.39
γ	mol^{-1} L	2532 ± 76	2770 ± 96	2616 ± 75	2778 ± 114
k_1	mol^{-1} L s^{-1}	2.50 × 10^6	2.42 × 10^6	2.48 × 10^6	2.54 × 10^6
$(1/k_2 + 1/k_3)^{-1}$	mol^{-1} L s^{-1}	3.56 × 10^5	1.67 × 10^6	3.72 × 10^5	6.09 × 10^5
$[H_2O_2]_{maxR}$	mol L^{-1}	0.000187	0.000184	0.000184	0.000176

Note: * Not significant ($p > 0.05$).

6.3.4. Stability of Coprinus *peroxidase*

6.3.4.1. Effect of pH

Stability of the crude and purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 against pH was investigated by incubating the enzyme solutions containing approximately 0.03 U mL^{-1} of active enzyme at various pH values for a 5-day period. The results are shown in Figure 6.5. In general, purified peroxidases were more stable than crude enzymes, except at pH 4 for the peroxidase from *Coprinus*

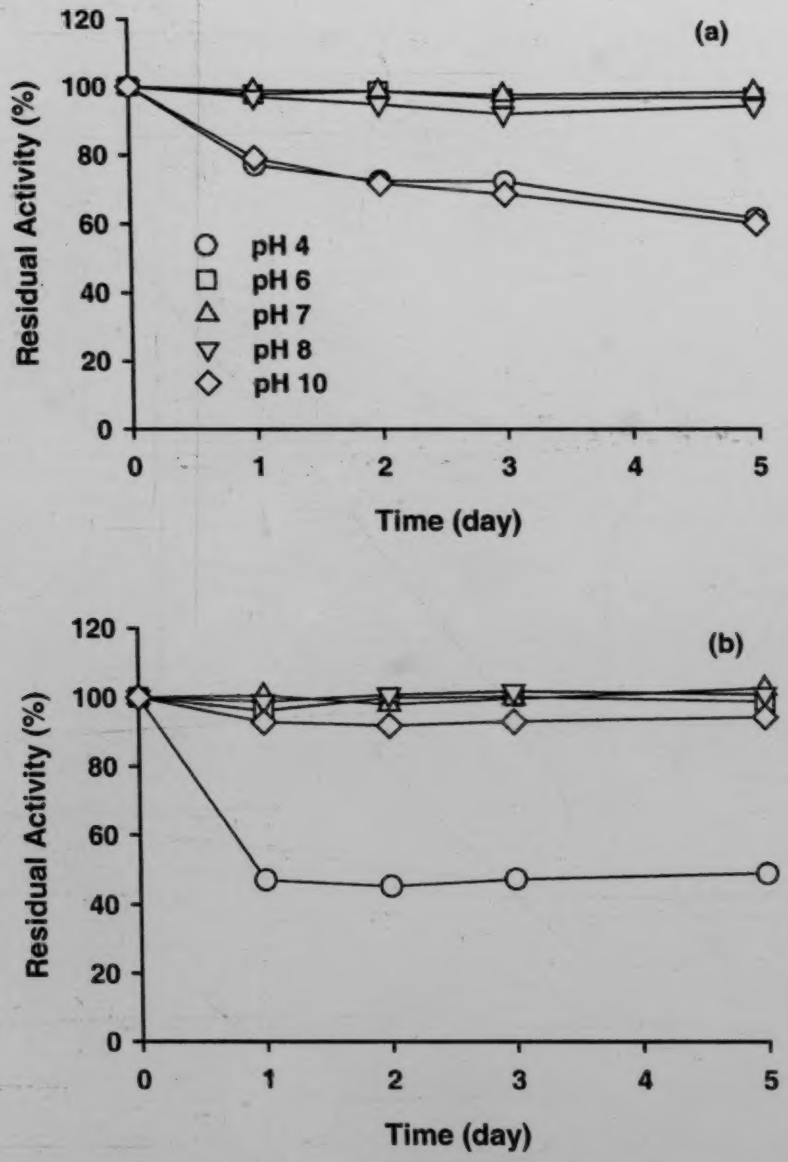
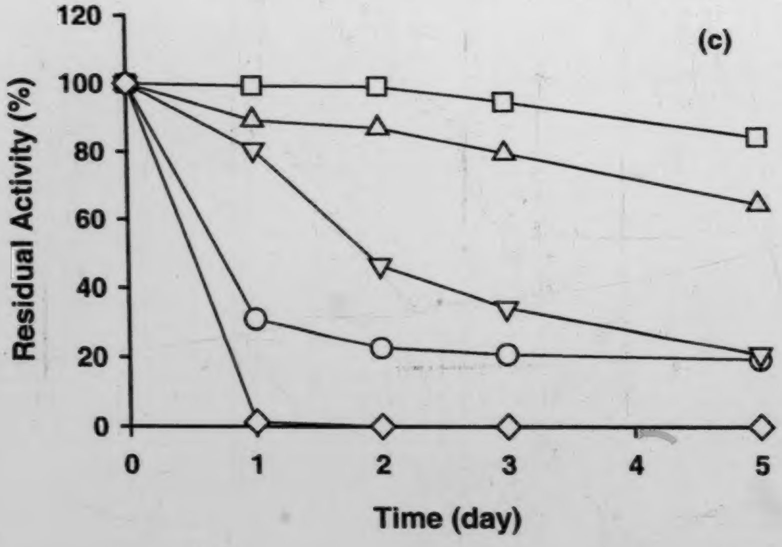


Figure 6.5. pH stability of the peroxidases from *Coprinus* sp. UAMH 10067l: (a) crude, (b) purified, and that from *C. cinereus* UAMH 4103: (c) crude, (d) purified at 25°C. Initial peroxidase activity was approximately 0.015 U mL^{-1}. Average values of two samples are shown. (See next page.)



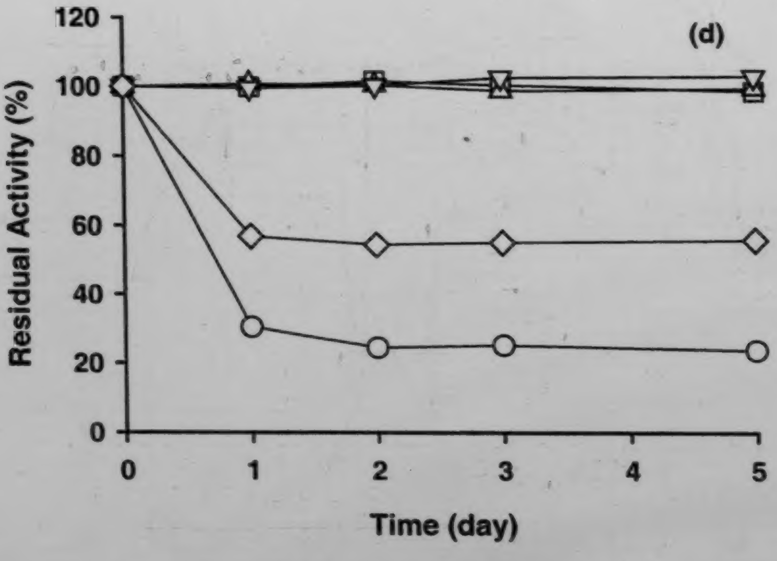


Figure 6.5. pH stability of the peroxidases from *Coprinus* sp. UAMH 10067l: (a) crude, (b) purified, and that from *C. cinereus* UAMH 4103: (c) crude, (d) purified at 25°C. Initial peroxidase activity was approximately 0.015 U mL^{-1}. Average values of two samples are shown (continued). Refer the previous page for legends.

sp. UAMH 10067. The peroxidase from *Coprinus* sp. UAMH 10067 was more stable at low and high pH than the enzyme from *C. cinereus* UAMH 4103. There was virtually no loss in peroxidase activity in the purified enzyme solutions at pH 6 to pH 8. At high and low pH, the peroxidase activity declined to a certain level within 1 day and stayed nearly constant over the remaining 4 days. On the other hand, the peroxidase activity slowly declined in the crude enzyme solutions during the incubation period.

6.3.4.2. Effect of temperature

Thermal stability of the *Coprinus* peroxidases at neutral pH was investigated by incubating the enzyme solution in phosphate buffer pH 7 for a 2-hour period at various temperatures ranging from 50°C to 70°C. The results for 50°C are shown in Figure 6.6. Crude peroxidase from *Coprinus* sp. UAMH 10067 was very stable at this temperature and retained more than 90% of its initial activity after 2 hours. The activity of crude peroxidase from *C. cinereus* UAMH 4103 dropped quickly over the first hour. Its rate of inactivation was slower, however, during the second hour. The purified peroxidases from both strains instantaneously lost some of their activity and were slowly inactivated further over time. The peroxidase from *Coprinus* sp. UAMH 10067 was also more stable than that from *C. cinereus* UAMH 4103 in purified form as well. These trends were also observed at more elevated temperatures (data not shown).

6.3.5. Batch treatment of aqueous phenol with* Coprinus *peroxidase

In order to compare the phenol removal efficiencies of the peroxidases evaluated in this study, buffered aqueous phenol was treated with various amounts of the crude and purified enzymes from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 at 25°C

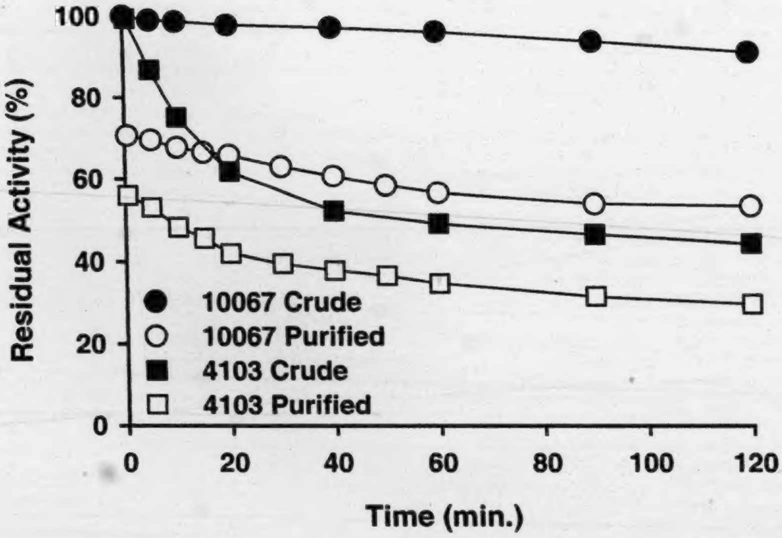


Figure 6.6. Thermal stability of the crude and purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 at 50°C at pH 7. Initial peroxidase activity was approximately 0.015 U mL^{-1}. Average values of two samples are shown.

and pH 7. The effect of the addition of PEG, which is a known protective agent of other peroxidases such as HRP and SBP (Nakamoto and Machida, 1992; Kinsley and Nicell, 2000), on the phenol removal was also studied. The results are shown in Figure 6.7.

The peroxidase from *Coprinus* sp. UAMH 10067 and that from *C. cinereus* UAMH 4103 performed nearly the same in the phenol removal experiments, regardless of the enzyme purity or PEG addition. Especially, in the presence of PEG, the enzyme requirements for 95% removal of 1.1 mM phenol were essentially identical for all four preparations of *Coprinus* peroxidases, and were 0.6 U mL^{-1}. In the absence of PEG, the enzyme requirements were substantially different between crude and purified *Coprinus* peroxidases: 0.75 U mL^{-1} for the crude enzyme from *Coprinus* sp. UAMH 10067, 0.9 U mL^{-1} for the crude enzyme from *C. cinereus* UAMH 4103, and 13.5 U mL^{-1} for the

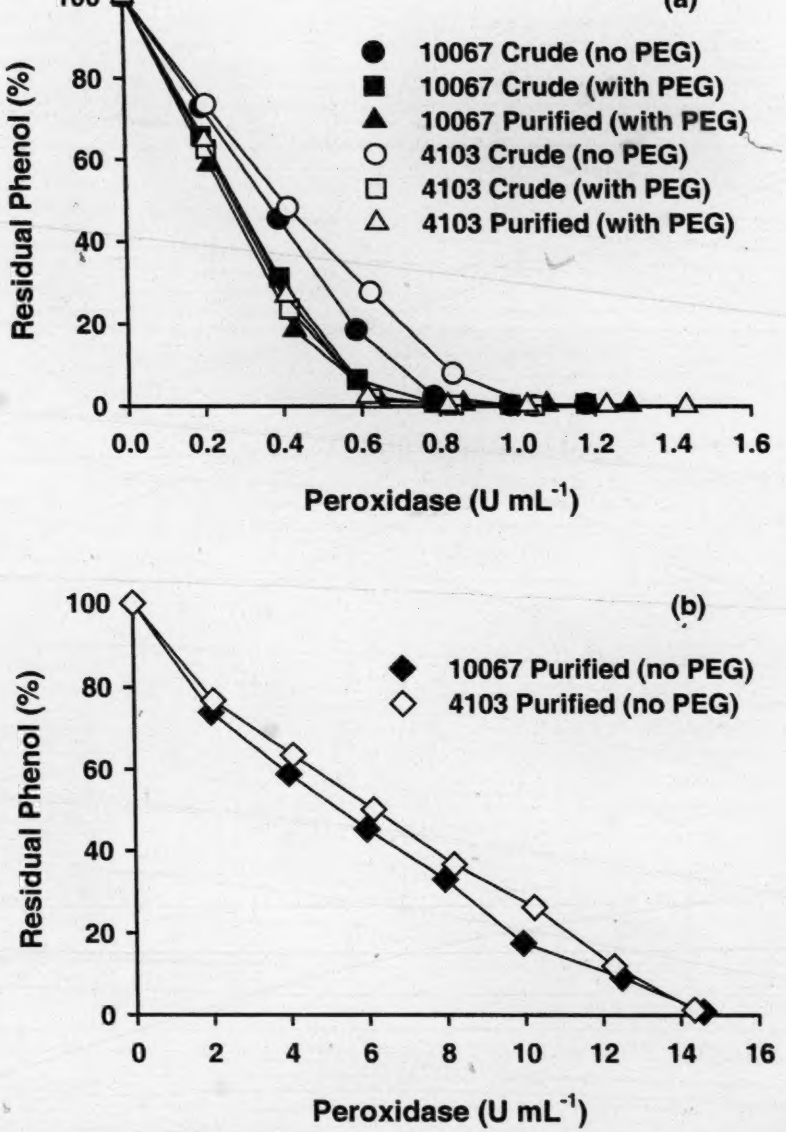


Figure 6.7. Aqueous phenol removal as a function of initial peroxidase activity using crude and purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 in the presence and absence of 200 mg L^{-1} PEG at 25°C at pH 7. ($[\text{phenol}]_0 = 1.1$ mM, $[H_2O_2]_0 = 1.5$ mM).

purified enzymes from both strains. The use of crude *Coprinus* peroxidases reduced the enzyme demand by 15 to 18 fold compared to the cases where purified enzymes were used. In addition, when PEG was added to the reaction mixture, the enzyme demand was reduced 23-fold compared to the case where purified enzymes were used.

Since the pH stability was different between the two *Coprinus* peroxidases evaluated in this study, the effect of pH on the phenol treatment was also studied. Purified enzymes with 200 mg L^{-1} PEG were used in this experiment in order to eliminate the purity effect of enzyme. As shown in Figure 6.8, the peroxidase from *Coprinus* sp. UAMH 10067 performed better at pH 9 than did the enzyme from *C. cinereus* UAMH 4103, especially where a larger amount of enzyme (1.2 U mL^{-1}) was applied to the reaction mixture. Both enzymes performed equally well at pH 6 to pH 8. The optimum pH for the phenol removal was observed at pH 7 for both enzymes.

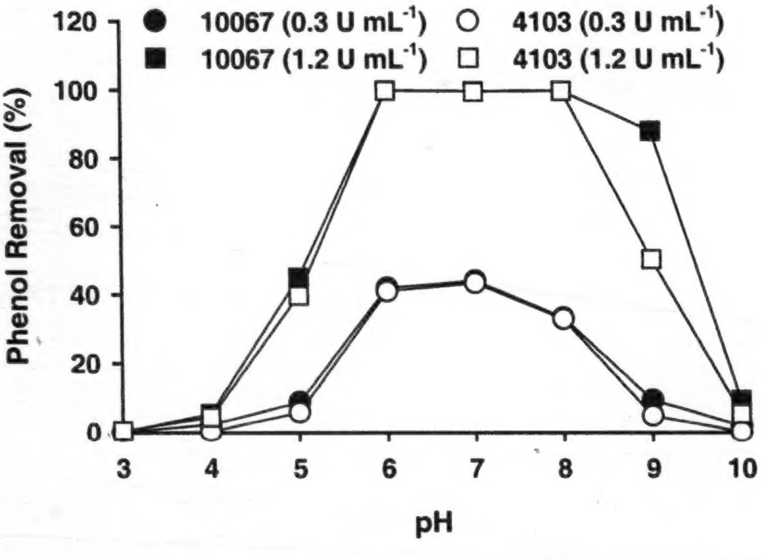


Figure 6.8. Effect of pH on the phenol removal using purified peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 at 25°C. ($[phenol]_0 = 1.1$ mM, $[H_2O_2]_0 = 1.5$ mM, $[PEG]_0 = 200$ mg L^{-1}).

The effect of reaction temperature on the phenol treatment was also studied by conducting the phenol removal experiments using two *Coprinus* peroxidases at pH 7 and 50°C with 200 mg L^{-1} of PEG. However, there was no marked difference between crude and purified peroxidases from both *Coprinus* species at this temperature (data not shown).

6.4. Discussion

In this study, fungal peroxidases from the two *Coprinus* species, including *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103, were purified by anion-exchange chromatography and size-exclusion chromatography. Based on MALDI-TOF MS, both enzymes had very similar molecular weights of about 36 kDa, which is slightly lower than previously reported value of 38 kDa for CIP from *C. cinereus* IFO 30114 and for ARP based on SDS-PAGE (Kjalke *et al.*, 1992). Since *Coprinus* peroxidases are glycoproteins (Morita *et al.*, 1988) and the SDS-PAGE molecular weight estimation is affected by the carbohydrate content of a glycoprotein, it is considered that the mass spectrometry used in this study gives a more accurate estimate of the molecular weight of *Coprinus* peroxidase. The minor band observed near 30 kDa in the samples of purified peroxidase from *C. cinereus* UAMH 4103 and commercial ARP in Figure 6.2 likely corresponds to the enzyme nicked at the carboxyl side of Asn-304, which was observed in ARP and CIP (Kjalke *et al.*, 1992; Kunishima *et al.*, 1993). It is known that the nicked peptides are linked with a disulphide bond of cystein residues (Kjalke *et al.*, 1992). There is no band near 30 kDa in the sample of the purified peroxidase from

Coprinus sp. UAMH 10067, although the band was present in the samples from earlier purification stages (data not shown). Thus, the fragmented enzyme might have been removed during the purification.

There were multiple forms of *Coprinus* peroxidase with slight variations in the molecular weight with an increment of 160 Da. This may imply that the degree of glycosylation is different among the isoenzymes. Morita *et al.* (1988) and Kjalke *et al.* (1992) also reported multiple isoenzymes of CIP from *C. cinereus* IFO 30116 and IFO 30114, respectively, that could be separated by affinity chromatography using Concanavalin A-Sepharose. Since the degree of glycosylation has no effect on the catalytic properties of *Coprinus* peroxidase (Kjalke *et al.*, 1992), further purification was not attempted in this study. The amino acid composition analyses (Table 6.2) indicate that the two peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 purified in this study and previously characterised CIP and ARP (Kjalke *et al.*, 1992; Baunsgaard *et al.*, 1993; Sawai-Hatanaka *et al.*, 1995) are very similar.

Identical pH optima for the peroxidase activity of the peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 were observed at pH 6. This is slightly different from the optimum pH for CIP using guaiacol as a substrate (pH 8; Andersen *et al.*, 1991) and from that using phenol as a substrate (pH 7.5; Masuda *et al.*, 2002). This discrepancy may be due to the difference in the assay procedures or conditions. As shown in Figure 6.3, purified *Coprinus* peroxidases were less active at pH 7 and pH 8 than crude enzymes, although the difference was relatively minor. In order to reduce the cost, enzyme purification will not likely be employed fully for the use of the enzyme in wastewater treatment. Thus, the higher activity of crude *Coprinus* peroxidase is considered beneficial for such a purpose. As compared to other peroxidases examined for phenolic wastewater treatment, *Coprinus* peroxidase has a broader pH-activity range than SBP (Wright and Nicell, 1999), but has a narrower range than HRP (Nicell *et al.*, 1993).

The relative activity versus hydrogen peroxide concentration plots shown in Figure 6.4 indicate that the two *Coprinus* peroxidases studied here have an identical catalytic property, at least under the conditions evaluated in this study, and that purification has no effect on the catalytic property. The model developed for plant peroxidases (Nicell and Wright, 1997) was applicable to the *Coprinus* peroxidase, and some kinetic parameters could be estimated successfully from the plots. The estimated k_1 value for *Coprinus* peroxidase in this study is in close agreement with the published values for CIP as well as ARP (Abelskov *et al.*, 1997; Buchanan and Han, 2000). The comparison of the kinetic parameters for *Coprinus* peroxidase to published values for SBP and HRP is presented in Table 6.5. Since there was no significant difference between the parameters for the two *Coprinus* peroxidases, the averaged values were calculated and are shown in Table 6.5.

Table 6.5. Comparison of kinetic parameters for *Coprinus* peroxidase, soybean peroxidase and horseradish peroxidase at pH 7.4 and 25°C.

Parameter	Unit	*Coprinus* peroxidase*	SBP†	HRP†
k_1	$mol^{-1}\ L\ s^{-1}$	2.49×10^6	1.1×10^6	2.3×10^6
$(1/k_2 + 1/k_3)^{-1}$	$mol^{-1}\ L\ s^{-1}$	7.52×10^5	2.6×10^3	3.7×10^4
$[H_2O_2]_{maxR}$	$mol\ L^{-1}$	0.000183	0.000331	0.000338

Note:

* Averaged values of four estimates in Table 4.

† From Nicell and Wright (1997).

As shown in Figure 6.1, k_1 is the rate constant for the hydrogen peroxide uptake by native peroxidase, k_2 and k_3 are the rate constants for the one-electron phenol oxidation by Compound I and Compound II, respectively. The comparison of k_1 and $(1/k_2 + 1/k_3)^{-1}$ values in Table 6.5 reveals that *Coprinus* peroxidase catalyses the phenol oxidation more than one to two orders of magnitude faster than HRP and SBP and exhibits similar or slightly faster hydrogen peroxide uptake. The $[H_2O_2]_{maxR}$ is smaller for *Coprinus* peroxidase than for HRP and SBP, which indicates that the former enzyme is more susceptible to the inhibition of peroxidase activity due to Compound III formation than are the latter ones.

Although the catalytic activities of the two *Coprinus* peroxidases investigated in this study were identical regardless of their purity, the stability of the two enzymes was substantially different, and the enzyme purification affected their stability as well. The peroxidase from *Coprinus* sp. UAMH 10067 is more stable at high temperature (50°C) and at alkaline pH (pH 10) than the enzyme from *C. cinereus* UAMH 4103. This may be an advantage of using the former enzyme for the wastewater treatment. The difference in the stability of the two *Coprinus* peroxidases may be due to the slight variations in the amino acid composition (Table 6.3) and in the molecular structure (*i.e.*, degree of glycosylation), although more comprehensive structural analysis will be needed to conclude this issue. In general, purification of *Coprinus* peroxidase enhanced the pH stability, although it had a detrimental effect on the thermal stability. Since crude or partly purified enzyme would likely be used for the wastewater treatment, the effect of purification or impurity on the enzyme stability is an important consideration. Thus, the higher thermal stability of crude *Coprinus* peroxidase is encouraging for such application. The lower pH stability of crude enzyme may be disadvantageous; however, the fungal peroxidase-catalysed phenol removal is a very fast process and requires a short retention time (minutes to a few hours) (Buchanan and Han, 2000). Therefore, the long-term pH stability demonstrated in Figure 6.5 is not likely important unless the enzyme were continuously used for longer incubation periods using such techniques as immobilisation.

The phenol removal experiments confirmed that the crude *Coprinus* peroxidase showed better phenol removal efficiency than purified enzyme, as was reported previously (Masuda *et al.*, 2001b; Ikehata and Buchanan, 2002), and PEG addition could enhance the phenol removal further (Figure 6.7). As there was no difference between the catalytic properties of the two *Coprinus* peroxidases studied (purified or crude), the enhanced performance of crude enzyme was likely due to the inhibition of peroxidase entrapment by phenol polymer products in the presence of biopolymers derived from the crude enzyme solution (Masuda *et al.*, 2001b). Because Masuda *et al.* (2001b) also demonstrated that the partial purification lowered the phenol removal efficiency of the CIP-catalysed treatment, PEG addition may be recommended depending on the operational requirements as it showed more consistent effect compared to those of fungal metabolites, and its dosage can be controlled easily.

Although there is virtually no difference in phenol removal efficiency between two *Coprinus* peroxidases at neutral pH, the peroxidase from *Coprinus* sp. UAMH 10067 performed better under weakly basic conditions (pH 9) than did the enzyme from *C. cinereus* UAMH 4103 (Figure 6.8). The broader pH range for the phenol removal may be another advantage of using the former enzyme for the wastewater treatment. Because the catalytic properties of these two enzymes were identical, the difference in the phenol removal at the alkaline pH is likely due to the difference in the pH stability (Figure 6.5).

The optimum pH for the phenol removal using *Coprinus* peroxidase determined in this study (pH 7) is close to one of the published values (pH 8; Kauffmann *et al.*, 1999), but different from the other published values (pH 9; Al-Kassim *et al.*, 1994b; Masuda *et al.*, 2001a; Masuda *et al.*, 2002) for crude and purified CIP from different sources. This discrepancy may be due to differences between the enzymes or to differences in the experimental and analytical methods.

The enzyme requirements of *Coprinus* peroxidases and previously studied plant peroxidases for the removal of 1 mM phenol in the presence or in the absence of PEG are summarised in Table 6.6. It can be seen that while the enzyme requirements vary drastically in the absence of PEG, they are similar in the presence of PEG among these enzymes. Although plant peroxidases, including HRP and SBP, are more efficient in phenol removal than *Coprinus* peroxidase in the presence of PEG, the use of crude *Coprinus* peroxidase may be justified when bulk production of this fungal enzyme is established. For that purpose, the optimisation of *Coprinus* peroxidase production by means of optimum medium development as well as semi-continuous production of the enzyme in larger bioreactors is currently underway.

6.5. Conclusion

Non-ligninolytic extracellular fungal peroxidases from two *Coprinus* species, *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103, were evaluated in this study as cost-effective alternatives to HRP for aqueous phenol treatment. The peroxidases obtained from the liquid culture filtrates of two *Coprinus* species were purified by anion-exchange chromatography, size-exclusion chromatography and FPLC. The purified peroxidases have very similar molecular and structural properties to *C. cinereus*

Table 6.6. Comparison of peroxidase requirement for the removal of 1 mM phenol at pH 7 and 25°C.

Enzyme	Enzyme Concentration ($U\ mL^{-1}$)		Removal
	Without PEG	With PEG	(%)
SBP [*]	0.9	0.6	> 95
SBP [†]	1.35	0.3	> 95
HRP [‡, ¶]	4.5	0.3	100
Crude *Coprinus* peroxidase UAMH 10067 [§, ¶]	0.75	0.6	> 95
Crude *Coprinus* peroxidase UAMH 4103 [§, ¶]	0.9	0.6	> 95
Purified *Coprinus* peroxidase [§, ¶]	13.5	0.6	> 95

Note:

[*] From Casa et al. (1999).

[†] From Kinsley and Nicell (2000).

[‡] From Ikehata and Buchanan (2002).

[§] This study.

[¶] Initial phenol concentration = 1.1 mM.

peroxidase studied previously. The two *Coprinus* peroxidases investigated in this study were catalytically identical. Whereas *Coprinus* peroxidase is catalytically faster than HRP and SBP in hydrogen peroxide uptake and phenol oxidation, the former enzyme is more susceptible to the inhibition by hydrogen peroxide than the latter ones. The peroxidase from *Coprinus* sp. UAMH 10067 was more stable at high pH and high temperature than the enzyme from *C. cinereus* UAMH 4103, which may be advantageous to the application of the former enzyme to the industrial wastewater treatment. The phenol removal efficiency of the *Coprinus* peroxidase was comparable to those of previously studied plant peroxidases at pH 7 and 25°C. Therefore, it can be suggested that the *Coprinus* peroxidases evaluated in this study have a great potential for application to phenolic wastewater treatment, when the bulk production of these fungal enzymes is established.

References

Abelskov, A. K., Smith, A. T., Rasmussen, C. B., Dunford, H. B. and Welinder, K. G. (1997). pH dependence and structural interpretation of the reactions of *Coprinus cinereus* peroxidase with hydrogen peroxide, ferulic acid, and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid). *Biochemistry* **36**(31): 9453-9463.

Al-Kassim, L., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1994a). Optimization of phenol removal by a fungal peroxidase from *Coprinus macrorhizus* using batch, continuous, and discontinuous semibatch reactors. *Enzyme and Microbial Technology* **16**: 120-124.

Al-Kassim, L., Taylor, K. E., Nicell, J. A., Bewtra, J. K. and Biswas, N. (1994b). Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxide from *Coprinus macrorhizus* in batch reactors. *Journal of Chemical Technology and Biotechnology* **61**: 179-182.

Andersen, M. B., Hsuanyu, Y., Welinder, K. G., Schneider, P. and Dunford, H. B. (1991). Spectral and kinetic properties of oxidized intermediates of *Coprinus cinereus* peroxidase. *Acta Chemica Scandinavica* **45**: 1080-1086.

Baunsgaard, L., Dalboge, H., Houen, G., Rasmussen, E. M. and Welinder, K. G. (1993). Amino acid sequence of *Coprinus macrorhizus* peroxidase and cDNA sequence encoding *Coprinus cinereus* peroxidase. A new family of fungal peroxidases. *European Journal of Biochemistry* **213**: 605-611.

Bennie, D. T. (1999). Review of the environmental occurrence of alkylphenols and alkylphenol ethoxylates. *Water Quality Research Journal of Canada* **34**(1): 79-122.

Buchanan, I. D. and Han, Y. S. (2000). Assessment of the potential of *Arthromyces ramosus* peroxidase to remove phenol from industrial wastewaters. *Environmental Technology* **21**: 545-552.

Buchanan, I. D. and Nicell, J. A. (1999). A simplified model of peroxidase-catalyzed phenol removal from aqueous solution. *Journal of Chemical Technology and Biotechnology* **74**: 669-674.

Caza, N., Bewtra, J. K., Biswas, N. and Taylor, K. E. (1999). Removal of phenolic compounds from synthetic wastewater using soybean peroxidase. *Water Research* **33**(13): 3012-3018.

Cooper, V. A. and Nicell, J. A. (1996). Removal of phenols from a foundry wastewater using horseradish peroxidase. *Water Research* **30**(4): 954-964.

Flock, C., Bassi, A. and Gijzen, M. (1999). Removal of aqueous phenol and 2-chlorophenol with purified soybean peroxidase and raw soybean hulls. *Journal of Chemical Technology and Biotechnology* **74**(4): 303-309.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (2001). Enzyme-catalyzed removal of phenol from refinery wastewater: Feasibility studies. *Water Environment Research* **73**(2): 165-172.

Ikehata, K. and Buchanan, I. D. (2002). Screening of *Coprinus* species for the production of extracellular peroxidase and evaluation of the enzyme for the treatment of aqueous phenol. *Environmental Technology* **23**(12): 1355-1368.

Ikehata, K., Buchanan, I. D. and Smith, D. W. (2003a). Treatment of oil refinery wastewater using crude *Coprinus cinereus* peroxidase and hydrogen peroxide. *Journal of Environmental Engineering and Science* (Accepted).

Ikehata, K., Buchanan, I. D. and Smith, D. W. (2003b). Extracellular peroxidase production by *Coprinus* species from urea treated soil. *Canadian Journal of Microbiology* (Submitted).

Karam, J. and Nicell, J. A. (1997). Potential application of enzymes in waste treatment. *Journal of Chemical Technology and Biotechnology* **69**: 141-153.

Kauffmann, C., Petersen, B. R. and Bjerrum, M. J. (1999). Enzymatic removal of phenols from aqueous solutions by *Coprinus cinereus* peroxidase and hydrogen peroxide. *Journal of Biotechnology* **73**: 71-74.

Kinsley, C. and Nicell, J. A. (2000). Treatment of aqueous phenol with soybean peroxidase in the presence of polyethylene glycol. *Bioresource Technology* **13**(2): 139-146.

Kjalke, M., Andersen, M. B., Schneider, P., Christensen, B., Schulein, M. and Welinder, K. G. (1992). Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. *Biochimica et Biophysica Acta* **1120**: 248-256.

Klibanov, A. M., Alberti, B. N., Morris, E. D. and Felshin, L. M. (1980). Enzymatic removal of toxic phenols and anilines from waste waters. *Journal of Applied Biochemistry* **2**: 414-421.

Klibanov, A. M., Tu, T.-M. and Scott, K. P. (1983). Peroxidase-catalyzed removal of phenols from coal-conversion waste water. *Science* **221**: 259-261.

Kunishima, N., Fukuyama, K., Wakabayashi, S., Sumida, M., Takaya, M., Shibano, Y., Amachi, T. and Matsubara, H. (1993). Crystallization and preliminary X-ray diffraction studies of peroxidase from a fungus *Arthromyces ramosus*. *PROTEINS: Structure, Function, and Genetics* **15**: 216-220.

Masuda, M., Sakurai, A. and Sakakibara, M. (2001a). Effect of reaction conditions on phenol removal by polymerization and precipitation using *Coprinus cinereus* peroxidase. *Enzyme and Microbial Technology* **28**: 295-300.

Masuda, M., Sakurai, A. and Sakakibara, M. (2001b). Effect of enzyme impurities on phenol removal by the method of polymerization and precipitation catalyzed by *Coprinus cinereus* peroxidase. *Applied Microbiology and Biotechnology* **57**: 494-499.

Masuda, M., Sakurai, A. and Sakakibara, M. (2002). Effect of temperature and pH on phenol removal using purified *Coprinus cinereus* peroxidase. *World Journal of Microbiology and Biotechnology* **18**: 739-743.

Morita, Y., Yamashita, H., Mikami, B., Iwamoto, H., Aibara, S., Terada, M. and Minami, J. (1988). Purification, crystallization, and characterization of peroxidase from *Coprinus cinereus*. *Journal of Biochemistry* **103**(4): 693-699.

Nakamoto, S. and Machida, N. (1992). Phenol removal from aqueous solutions by peroxidase-catalyzed reaction using additives. *Water Research* **26**(1): 49-54.

National Toxicology Program (2003). *Chemical Health and Safety Information*. National Toxicology Program, National Institute of Environmental Health Sciences, National Institute of Health, U.S.A., Accessed on July 30th, 2003. URL: http://ntp-server.niehs.nih.gov/Main_Pages/Chem-HS.html.

Nicell, J. A., Bewtra, J. K., Biswas, N., Pierre, C. C. S. and Taylor, K. E. (1993). Enzyme catalyzed polymerization and precipitation of aromatic compounds from aqueous solution. *Canadian Journal of Civil Engineering* **20**: 725-735.

Nicell, J. A. and Wright, H. (1997). A model of peroxidase activity with inhibition by hydrogen peroxide. *Enzyme and Microbial Technology* **21**: 302-310.

Sakurai, A., Kawamoto, S., Abarca, J. F. and Sakakibara, M. (2002). Peroxidase production by *Coprinus cinereus* using rotating disk contactor. *Biochemical Engineering Journal* **10**: 47-53.

Sawai-Hatanaka, H., Ashikari, T., Tanaka, Y., Asada, Y., Nakayama, T., Minakata, H., Kunishima, N., Fukuyama, K., Yamada, H., Shibano, Y. and Amachi, T. (1995). Cloning, sequencing, and heterologous expression of a gene coding for *Arthromyces ramosus* peroxidase. *Bioscience, Biotechnology, and Biochemistry* **59**(7): 1221-1228.

Servos, M. R. (1999). Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol ethoxylates. *Water Quality Research Journal of Canada* **34**(1): 123-177.

Shinmen, Y., Asami, S., Amachi, T., Shimizu, S. and Yamada, H. (1986). Crystallization and characterization of an extracellular fungal peroxidase. *Agricultural and Biological Chemistry* **50**(1): 247-249.

Singleton, D. W. and Khan, S. A. (2003). Xenoestrogen exposure and mechanisms of endocrine disruption. *Frontiers in Bioscience* **8**: S110-S118.

US EPA (2002). *2000 Toxics Release Inventory (TRI) Public Data Release Report.* EPA-260-R-02-003, United States Environmental Protection Agency, Washington, DC.

Villalobos, D. A. and Buchanan, I. D. (2002). Removal of aqueous phenol by *Arthromyces ramosus* peroxidase. *Journal of Environmental Engineering and Science* **1**: 65-73.

Wagner, M. and Nicell, J. A. (2001a). Peroxidase-catalyzed removal of phenols from a petroleum refinery wastewater. *Water Science and Technology* **43**(2): 253-260.

Wagner, M. and Nicell, J. A. (2001b). Treatment of a foul condensate from Kraft pulping with horseradish peroxidase and hydrogen peroxide. *Water Research* **35**(2): 485-495.

Wright, H. and Nicell, J. A. (1999). Characterization of soybean peroxidase for the treatment of aqueous phenol. *Bioresource Technology* **70**(1): 69-79.

Wu, Y., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997). Comparison of additives in the removal of phenolic compounds by peroxidase-catalyzed polymerization. *Water Research* **31**(11): 2699-2704.

Yao, P.-X., Matsumoto, N., Tsujimura, H., Takaya, M. and Toda, K. (1995). Enhanced production of peroxidase in continuous culture of *Arthromyces ramosus* by selective bleeding of mycelium. *Journal of Fermentation and Bioengineering* **79**(4): 307-312.

Zhang, G. and Nicell, J. A. (2000). Treatment of aqueous pentachlorophenol by horseradish peroxidase and hydrogen peroxide. *Water Research* **34**(5): 1629-1637.

Chapter 7. Optimisation of Extracellular Fungal Peroxidase Production by Two *Coprinus* Species[*]

7.1. Introduction

An inky-cup mushroom, *Coprinus cinereus*, is known to produce a non-specific extracellular peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7), which is a valuable biocatalyst for such potential applications as clinical assays, biochemical synthesis, biochemical sensing, and bioremediation and wastewater treatment (Morita *et al.*, 1988; Nakayama and Amachi, 1999). Enzymatic removal of phenols from industrial wastewater is considered as a promising environmental application of *Coprinus* peroxidase and has been actively investigated for years (Al-Kassim *et al.*, 1994; Kauffmann *et al.*, 1999; Masuda *et al.*, 2001; Ikehata and Buchanan, 2002; Ikehata *et al.*, 2003a). Although the effectiveness of *Coprinus* peroxidase catalysed treatment of phenolic wastewater has been proven, only a few studies had been reported to date regarding the peroxidase production by *C. cinereus* and related fungi (Tsujimura *et al.*, 1994; Yao *et al.*, 1995; Sakurai *et al.*, 2002), and none has been done on the medium development to maximise the peroxidase production by this fungi.

Our recent fungal survey demonstrated the peroxidase production by other *Coprinus* species such as *C. lagopus* UAMH 7499, *C. echinosporus* NBRC 30630, *C. macrocephalus* NBRC 30117 and several unidentified *Coprinus* species (Ikehata and Buchanan, 2002; Ikehata *et al.*, 2003b). Among the fungi tested in the survey, three strains of *C. cinereus* NBRC 30114 (IFO 30114), UAMH 4103 and UAMH 7907, as well as a newly isolated *Coprinus* sp. UAMH 10067, were found to be promising peroxidase producers for large scale production and characterisation. Of these *Coprinus* species, we are particularly interested in the peroxidase produced by *Coprinus* sp. UAMH 10067, because this enzyme is apparently more stable at high temperature (50°C) than the enzyme produced by *C. cinereus*, although the enzyme productivity was lower than the latter fungus (Ikehata *et al.*, 2003b). High thermal stability of an enzyme is generally preferable, and especially beneficial for its application to industrial wastewater treatment, because the temperature of industrial wastewaters tend to be higher than room temperature and tends to accelerate enzyme inactivation. To obtain the bulk enzyme for purification and further characterisation, as well as to lower the enzyme cost for the large-scale production and application, the optimisation of peroxidase production by the *Coprinus* species is required.

---

[*] A version of this chapter (by Ikehata, K., Pickard, M.A., Buchanan, I.D. and Smith, D.W.) has been submitted to the *Journal of Industrial Microbiology and Biotechnology* in July 2003.

Therefore, in this study, the optimum growth conditions for peroxidase production by *Coprinus* sp. UAMH 10067 were explored through the following three steps: 1) the determination of best carbon and nitrogen sources, 2) the screening step of important factors with a 2 level 7 factor (resolution IV; 2^{7-3}_{IV}) fractional factorial design, and 3) the optimisation step with a 2-factor central composite design. The optimum growth conditions for *C. cinereus* UAMH 4103 were also determined based in part on the outcome of the screening experiment conducted for the former fungus and a separate optimisation experiment with another central composite design. The results of the optimisation experiments were analysed by regression, and response surfaces were constructed to visualise the optimum growth conditions for each fungus.

7.2. Materials and methods

7.2.1. Fungi and general culture conditions

Coprinus sp. UAMH 10067 and *C. cinereus* UAMH 4103 were obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, Alberta, Canada. Stock cultures of these fungi were grown periodically on potato dextrose agar (PDA; Difco brand, Becton Dickinson, Sparks, Maryland) at 25°C for 7 to 10 days and stored at 4°C. Seed cultures for liquid fermentation were grown on PDA plates at 25°C for 7 to 10 days prior to the experiment.

Inocula were prepared by homogenising approximately 20 cm^2 of surface mycelium from a seed culture plate in 100 mL of liquid medium in a Sorvall Omnimixer (Sorvall, Norwalk, Connecticut). Four millilitres of homogenised mycelium were inoculated in 500 mL Erlenmeyer flasks containing 150 mL of a liquid medium and cultivated on a rotary shaker at 200 rpm and 27 ± 1°C. In the case of the screening and optimisation experiments, 1 mL samples were taken daily using sterilised pipettes for peroxidase activity assay, while flasks were harvested periodically in the case of confirmation growth experiments for medium pH, residual glucose concentration and biomass weight determinations, in addition to the enzyme assay. For biomass weight determination, fungal biomass was separated from the liquid medium by centrifugation at 2 000 *g* for 30 minutes and washed with pure water generated with Elgastat Maxima Water Purification System (Elga Ltd., High Wycombe, Bucks, England), dried in an oven at 70°C overnight and weighed. Supernatants were analysed for peroxidase activity, residual glucose and medium pH.

7.2.2. Experimental designs for the optimisation of **Coprinus** *peroxidase production*

7.2.2.1. Effects of carbon and nitrogen sources

The YM broth (Ikehata and Buchanan, 2002) containing 1% glucose, 0.5% Bacto® peptone (simply referred as peptone in the following), 0.3% yeast extract and 0.3% malt extract (all ingredients are Difco brand) was used as the basal medium for *Coprinus* peroxidase production and modified through the study. Prior to the screening and optimisation experiments, various sugars, complex nitrogen sources and inorganic nitrogen compounds listed in Table 7.1 were examined to determine the best carbon and nitrogen sources to support the peroxidase production. Concentrations of the carbon and nitrogen sources were fixed to 1% and 0.5%, respectively. All growth media were prepared in triplicate.

7.2.2.2. Screening of important factors

There are a number of factors in the growth conditions that may affect *Coprinus* peroxidase production. To screen these factors can be tedious if each factor is examined individually in conventional experimental designs. To reduce the number of screening experiments for increased efficiency, a 2 level 7 factor fractional factorial design (resolution IV; 2^{7-3}_{IV}) was employed. This design is a 1/8 (= 2^{-3}) fraction of a 2^7 full factorial design. Resolution IV here implies that no main effects are confounded with other main effects or two-factor interactions, although the two-factor interactions are confounded with other two-factor interactions. The main effects and interactions can be estimated using regression analysis (Berthouex and Brown, 2002). More than two-factor interaction effects cannot be estimated independently; however, these effects are often less important. Also, the primary focus of the screening experiment was to identify the

Table 7.1. Effect of carbon and nitrogen sources on the peroxidase production by *Coprinus* sp. UAMH 10067. Initial medium pH was adjusted at 6.2.

	Compound	Maximum peroxidase activity ($U\ mL^{-1}$)
Carbon source (1%) (0.5% peptone as a N source)	Glucose	10.7 ± 1.4
	Fructose	10.4 ± 0.6
	Xylose	2.2 ± 1.5
	Maltose	0.9 ± 0.3
	Lactose	0.6 ± 0.1
	Sucrose	0.4 ± 0.2
	Galactose	0.2 ± 0.1
	Starch	0.4 ± 0.2
Nitrogen source (0.5%) (1% glucose as a C source)	Casitone	12.3 ± 1.6
	Peptone	10.1 ± 2.3
	Tryptone	7.9 ± 0.4
	Phytone	6.1 ± 1.1
	$NaNO_3$	0.9 ± 0.3
	Urea	0.4 ± 0.1
	Yeast Extract (0.8% in total)	7.6 ± 1.3

Note: Other medium ingredients include 0.3% yeast extract and 0.3% malt extract. Mean and standard deviation are presented.

main effects of individual factors, which will be optimised further in subsequent experiments.

The factors examined in the screening experiment include: 1) glucose concentration, 2) nitrogen source concentration, 3) yeast extract concentration, 4) malt extract concentration, 5) type of nitrogen source (peptone or casitone), 6) addition of ferrous sulphate ($FeSO_4$) and 7) initial medium pH. The actual levels of these factors are summarised in Table 7.2. In order to estimate the sample variances, all runs were replicated.

Table 7.2. 2^{7-3}_{IV} Fractional factorial design for the screening experiment.

#	Glucose (%) **1**	N Source (%) **2**	Yeast Extract (%) **3**	Malt Extract (%) **4**	Type of N Source **5 = 123**	$FeSO_4$ Addition[*] **6 = 234**	pH **7 = 134**
1	1	0.5	0.3	0.3	Peptone	no	5.5
2	2	0.5	0.3	0.3	Casitone	no	6.5
3	1	1.0	0.3	0.3	Casitone	yes	5.5
4	2	1.0	0.3	0.3	Peptone	yes	6.5
5	1	0.5	0.6	0.3	Casitone	yes	6.5
6	2	0.5	0.6	0.3	Peptone	yes	5.5
7	1	1.0	0.6	0.3	Peptone	no	6.5
8	2	1.0	0.6	0.3	Casitone	no	5.5
9	1	0.5	0.3	0.6	Peptone	yes	6.5
10	2	0.5	0.3	0.6	Casitone	yes	5.5
11	1	1.0	0.3	0.6	Casitone	no	6.5
12	2	1.0	0.3	0.6	Peptone	no	5.5
13	1	0.5	0.6	0.6	Casitone	no	5.5
14	2	0.5	0.6	0.6	Peptone	no	6.5
15	1	1.0	0.6	0.6	Peptone	yes	5.5
16	2	1.0	0.6	0.6	Casitone	yes	6.5

Note: Bold letters indicate the identity of the factor.

[*] $FeSO_4$ was added to the final concentration of 2 mg L^{-1} in "yes" flasks.

7.2.2.3. Optimisation of glucose and peptone concentrations

Based on the results of the above screening experiment, the concentrations of glucose and peptone were chosen for in depth optimisation of peroxidase production by two *Coprinus* species: *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103. The optimisation experiments were conducted using a 2-factor central composite design. The design matrices of the optimisation experiments for these fungi are presented in Tables

7.3 and 7.4. A series of confirmation runs (A to D in Tables 7.3 and 7.4) were conducted separately from the original runs (#1 to #9) to test the reproducibility of the responses. All runs were conducted in triplicate.

Table 7.3. Design matrix and the response (maximum peroxidase activity in U mL^{-1}) of the central composite design for the optimisation of peroxidase production by *Coprinus* sp. UAMH 10067.

	Actual Level		Coded Level		Response		
#	Glucose (%)	Peptone (%)	Glucose	Peptone	Average	Std. Dev.	Modelled
1	2	0.7	-1	-1	21.3	0.2	23.5
2	3	0.7	1	-1	35.1	0.8	29.2
3	2	1.3	-1	1	21.2	2.3	21.6
4	3	1.3	1	1	25.5	1.0	25.9
5	1.79	1	-1.41	0	28.5	2.3	26.1
6	3.21	1	1.41	0	19.7	1.3	19.1
7	2.5	0.58	0	-1.41	30.3	1.2	29.4
8	2.5	1.42	0	1.41	26.5	2.6	25.7
9	2.5	1	0	0	26.9	1.4	30.1
10*	2.0	0.5	-1	-1.67	21.3	0.9	21.8
11*	3.0	0.5	1	-1.67	26.8	1.9	28.0
A†	1.8	0.8	-1.4	-0.67	21.6	1.3	19.2
B†	2.3	0.8	-0.4	-0.67	27.7	1.1	28.7
C†	2.8	0.8	0.6	-0.67	31.3	1.6	30.7
D†	3.3	0.8	1.6	-0.67	20.5	1.1	25.1

Note:

* Additional runs for the construction of response surface.

† Additional runs for the confirmation of repeatability.

The responses (maximum peroxidase activity) from the composite design experiments with two *Coprinus* species were analysed and modelled with regression, and the modelled response surfaces were plotted. The optimum glucose and peptone concentrations, which could be derived either from the plots or from the model equations, were subsequently used in the confirmation growth experiments.

Table 7.4. Design matrix and the response (maximum peroxidase activity in U mL^{-1}) of the central composite design for the optimisation of peroxidase production by *C. cinereus* UAMH 4103.

	Actual Level		Coded Level		Response		
#	Glucose (%)	Peptone (%)	Glucose	Peptone	Average	Std. Dev.	Modelled
1	2	0.5	-1	-1	30.6	2.8	30.2
2	3	0.5	1	-1	39.2	3.4	37.3
3	2	1.3	-1	1	60.5	2.6	57.1
4	3	1.3	1	1	73.1	1.9	69.1
5	1.79	0.9	-1.41	0	42.2	1.0	42.5
6	3.21	0.9	1.41	0	56.9	1.1	56.0
7	2.5	0.33	0	-1.41	-[*]	-[*]	26.9
8	2.5	1.47	0	1.41	63.6	3.3	68.3
9	2.5	0.9	0	0	58.6	3.1	58.4
A[†]	1.8	0.8	-1.4	-0.25	36.6	1.4	39.2
B[†]	2.3	0.8	-0.4	-0.25	55.4	0.9	51.9
C[†]	2.8	0.8	0.6	-0.25	50.3	1.7	55.4
D[†]	3.3	0.8	1.6	-0.25	47.9	1.3	49.8

Note:

[*] No data available due to contamination.

[†] Additional runs for the confirmation of repeatability.

7.2.3. Enzyme and chemical assays

Peroxidase activity was determined by a colourimetric assay using phenol and 4-aminoantipyrine (Fisher Scientific Canada, Edmonton, Alberta, Canada) as described previously (Ikehata and Buchanan, 2002). Glucose concentration was determined using a glucose oxidase/peroxidase method according to the glucose (GO) assay kit technical bulletin (Sigma).

7.2.4. Chemicals

All chemicals were of reagent grade and were purchased either from Sigma-Aldrich Canada (Oakville, Ontario, Canada) or from Fisher Scientific Canada. Phytone (BBL brand) and all other medium ingredients (Difco brand) manufactured by Becton Dickinson were purchased either from Fisher Scientific Canada or directly from the manufacturer.

7.3. Results

7.3.1. Effects of carbon and nitrogen sources on the peroxidase production by **Coprinus** *species*

Eight carbon sources and 7 nitrogen sources were evaluated for the growth of *Coprinus* sp. UAMH 10067 and its peroxidase production. The maximum peroxidase activity recorded for each culture medium is presented in Table 7.1. As a carbon source, glucose and fructose supported the highest peroxidase production, while other sugars, including xylose, maltose, lactose, sucrose and galactose, and starch suppressed both growth (not quantified) and peroxidase production. Glucose was selected as a carbon source for the further experiments because of its availability and cost compared to fructose.

It was shown that complex nitrogen sources, including casitone, peptone, tryptone, phytone and yeast extract, generally supported peroxidase production by *Coprinus* sp. UAMH 10067 better than the simple nitrogenous compounds, sodium nitrate and urea. Casitone and peptone supported the highest peroxidase production among the nitrogen sources tested. The average maximum peroxidase activity was slightly higher in the medium containing casitone than in that containing peptone, although there was no statistical difference ($p = 0.24$ with independent t-test, d.f. = 4). In order to clarify which nitrogen source was better to support *Coprinus* peroxidase production, casitone and peptone were selected for further investigation in the screening experiment.

7.3.2. Screening of important factors for **Coprinus** *peroxidase production*

The effects of seven factors of culture conditions, including concentrations of glucose, nitrogen source, yeast extract and malt extract; type of nitrogen source (peptone or casitone), addition of ferrous sulphate, and initial pH of the medium, were tested in a 2 level fractional factorial screening experiment for the peroxidase production by *Coprinus* sp. UAMH 10067. The responses of the screening experiments expressed as maximum peroxidase activities are summarised in Table 7.5. The responses were log-transformed in order to stabilise the variance. The coded matrix used in the experimental design and analysis is also shown in the table.

The fractional factorial experiment utilised in this study provided information to estimate 16 parameters, including an average, 7 main effects and 8 interactions. In order to estimate these parameters, the following regression model equation was applied:

$$\begin{aligned}\eta = {} & \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_6 x_6 + \beta_7 x_7 + \beta_{12} x_1 x_2 \\ & + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{15} x_1 x_5 + \beta_{16} x_1 x_6 + \beta_{17} x_1 x_7 + \beta_{24} x_2 x_4 \\ & + \beta_{1234567} x_1 x_2 x_3 x_4 x_5 x_6 x_7 \qquad (7\text{-}1)\end{aligned}$$

Table 7.5. Design matrix and the response (maximum peroxidase activity in U mL^{-1}) of the fractional factorial experiment.

																Response			
#	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**12**	**13**	**14**	**15**	**16**	**17**	**24**	**1234567**	#1	# 2	Ave.	Ln*
1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	-1	11.4	10.6	11.0	2.40
2	1	-1	-1	-1	1	-1	1	-1	-1	-1	1	-1	1	1	1	31.2	33.7	32.4	3.48
3	-1	1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	-1	1	10.8	11.5	11.2	2.41
4	1	1	-1	-1	-1	1	1	1	-1	-1	-1	1	1	-1	-1	30.0	28.3	29.2	3.37
5	-1	-1	1	-1	1	1	1	1	-1	1	-1	-1	-1	1	-1	10.1	8.7	9.4	2.24
6	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	-1	1	1	33.5	44.8	39.1	3.66
7	-1	1	1	-1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	10.5	7.8	9.1	2.20
8	1	1	1	-1	1	-1	-1	1	1	-1	1	-1	-1	-1	-1	21.4	28.5	24.9	3.21
9	-1	-1	-1	1	-1	1	1	1	1	-1	1	-1	-1	-1	1	13.2	10.5	11.8	2.46
10	1	-1	-1	1	1	1	-1	-1	-1	1	1	1	-1	-1	-1	33.2	27.7	30.4	3.41
11	-1	1	-1	1	1	-1	1	-1	1	-1	-1	1	-1	1	-1	10.8	14.4	12.6	2.52
12	1	1	-1	1	-1	-1	-1	1	-1	1	-1	-1	-1	1	1	19.7	20.9	20.3	3.01
13	-1	-1	1	1	1	-1	-1	1	-1	-1	-1	1	1	-1	1	12.7	15.5	14.1	2.64
14	1	-1	1	1	-1	-1	1	-1	1	1	-1	-1	1	-1	-1	33.1	40.0	36.5	3.59
15	-1	1	1	1	-1	1	-1	-1	-1	-1	1	-1	1	1	-1	13.5	14.1	13.8	2.62
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20.4	24.6	22.5	3.11

Note: Bold letters indicate the identity of the factor (see Table 7.2). The defining relation is **I** = **1234567**.

* Natural log of averaged response.

where the x_1 to x_7 are the levels of the seven experimental variables and the β's are regression coefficients that indicate the magnitude of the effects of each of the variables and the interactions of variables. These coefficients were estimated using the method of least squares with the following form of the model:

$$\begin{aligned} y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 + b_5 x_5 + b_6 x_6 + b_7 x_7 + b_{12} x_1 x_2 + b_{13} x_1 x_3 \\ + b_{14} x_1 x_4 + b_{15} x_1 x_5 + b_{16} x_1 x_6 + b_{17} x_1 x_7 + b_{24} x_2 x_4 \\ + b_{1234567} x_1 x_2 x_3 x_4 x_5 x_6 x_7 + e \end{aligned} \tag{7-2}$$

where *e* is the residual error and *b*'s are estimates of coefficients. The estimated coefficients are presented in Table 7.6 with standard error and 95% confidence interval of true effect. For general procedures of statistical data analysis, please consult Berthouex and Brown (2002) or Box *et al.* (1978).

Table 7.6. Estimated main effects and two-factor interactions for the fractional factorial experiment.

	Factors	Confounded Factors	Estimated Effect	Estimated Standard Error	95% Confidence Interval of True Effect	Significant?
b_0	Average	-	2.896	0.024	0.051	Yes
b_1	**1**	**235 = 267 = 347**	1.835	0.068	0.144	Yes
b_2	**2**	**135 = 167 = 346**	-0.356	0.068	0.144	Yes
b_3	**3**	**125 = 147 = 246**	0.050	0.068	0.144	No
b_4	**4**	**137 = 236 = 357**	0.103	0.068	0.144	No
b_5	**5**	**123 = 146 = 347**	-0.075	0.068	0.144	No
b_6	**6**	**127 = 145 = 234**	0.060	0.068	0.144	No
b_7	**7**	**126 = 134 = 345**	-0.096	0.068	0.144	No
b_{12}	**12**	**35 = 67**	-0.365	0.068	0.144	Yes
b_{13}	**13**	**25 = 47**	0.096	0.068	0.144	No
b_{14}	**14**	**37 = 56**	-0.397	0.068	0.144	Yes
b_{15}	**15**	**23 = 46**	-0.138	0.068	0.144	No
b_{16}	**16**	**27 = 45**	0.070	0.068	0.144	No
b_{17}	**17**	**26 = 34**	0.230	0.068	0.144	Yes
b_{24}	**24**	**36 = 57**	-0.066	0.068	0.144	No
$b_{1234567}$	**1234567**	-	-0.098	0.068	0.144	No

Note: Bold letters indicate the identity of the factor (see Table 2). Effects are expressed in the natural log scale.

As shown in Table 7.6, each main effect was confounded with three three-factor interactions, for example, the main effect of glucose concentration (**1**) was confounded with interaction of nitrogen source concentration, yeast extract concentration, and type of nitrogen source (**235**), that of nitrogen source concentration, addition of ferrous sulphate and initial medium pH (**267**) and that of yeast extract concentration, malt extract concentration and initial medium pH (**347**). Similarly, each two-factor interaction was confounded with two two-factor interactions.

Among the seven main effects and eight interactions shown in Table 7.6, the main effects of glucose concentration (**1**) and nitrogen source concentration (**2**) were significant, and the two-factor interactions of glucose concentration and nitrogen source concentration (**12**), of glucose concentration and malt extract concentration (**14**), and of glucose concentration and pH (**17**) were significant for the production of peroxidase by *Coprinus* sp. UAMH 10067, although the two-factor interactions were confounded with other two-factor interactions, which makes the interpretation of these two-factor interaction effects complicated. In this study, because the major objective of screening study was to determine which factors were important for the production of *Coprinus* peroxidase, and no main effects other than those of glucose and nitrogen source concentrations proved to be significant, these two factors were selected for further optimisation. Other factors were fixed at the original levels or conditions: 0.3% yeast extract, 0.3% malt extract, peptone as a nitrogen source, and no ferrous sulphate addition. Initial medium pH was unadjusted unless it deviated from the tested range, from 5.5 to 6.5.

7.3.3. Optimisation of glucose and peptone concentrations for **Coprinus** *peroxidase production*

From the screening experiment result (Table 7.6), it was observed that the medium glucose concentration showed a major positive effect (+1.835) on the peroxidase production by *Coprinus* sp. UAMH 10067, and the concentration of nitrogen source showed a minor negative effect (-0.356). Therefore, it was speculated that the optimum glucose concentration for the *Coprinus* peroxidase production would be found within the range tested in the screening experiment (1% to 2%) or higher, and that of peptone concentration would be found within the range of 0.5% to 1% or lower. Since the interaction effect of these two factors was also observed in the screening experiment (-0.365), these factors needed to be evaluated with a factorial experimental design. Thus, a 2-level factorial design (coded levels of -1 and +1) augmented with a center point (0) and star points (-1.414 and +1.414), also called a central composite design, was chosen in the optimisation experiment for the *Coprinus* peroxidase production (Tables 7.3 and 7.4). This design is based on a quadratic model that may be relevant to the responses of multifactor experiments (Berthouex and Brown, 2002). This design covers a relatively wide experimental region, which is also a favourable feature for optimisation experiments. Based on preliminary test results, slightly different actual levels of factors were chosen for two *Coprinus* species, *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103, as shown in Tables 7.3 and 7.4, respectively. The responses of the composite design experiments were also presented in Tables 7.3 and 7.4.

In order to analyse the data, the following quadratic model was applied:

$$\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 \qquad (7\text{-}3)$$

Similar to the case of the screening experiment, the regression coefficients were estimated using the method of least squares with the following form of the model:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + e \qquad (7\text{-}4)$$

where *e* is the residual error. In the case of *Coprinus* sp. UAMH 10067, since the solved model diverged and did not give an optimum point within the experimental region with the original runs (#1 to #9 in Table 7.3), additional runs (#10 and #11) were conducted to cover an extended region. Another set of additional runs (A to D in Tables 7.3 and 7.4) was also conducted for both strains of *Coprinus* species to check the repeatability of the experiments. The regression models were solved with and without the additional data, and the estimates and standard errors of the coefficients are presented in Table 7.7.

Table 7.7. Estimated model coefficients calculated from the results of the central composite design experiments. Standard errors of the estimated coefficients are also shown.

Estimated Model Coefficient	UAMH 4103 (a)	UAMH 4103 (b)	UAMH 10067 (a)	UAMH 10067 (b)
b_0	58.6 ± 2.1	58.4 ± 1.4	29.5 ± 1.2	30.2 ± 1.0
b_1	5.3 ± 0.7	4.7 ± 0.6	3.4 ± 0.5	2.5 ± 0.5
b_2	14.6 ± 0.9	14.7 ± 1.0	-1.3 ± 0.5	-1.3 ± 0.5
b_{12}	1.0 ± 1.0*	1.2 ± 1.1*	-0.8 ± 0.5*	-0.4 ± 0.6*
b_{11}	-3.6 ± 1.2	-4.6 ± 0.8	-2.8 ± 0.8	-3.8 ± 0.6
b_{22}	-6.0 ± 1.4	-5.4 ± 1.4	-1.3 ± 0.6	-1.3 ± 0.5

Note: The model coefficients were estimated: (a) without additional runs and (b) with additional runs (A to D in Tables 3 and 4). * Not significant ($p > 0.05$).

All estimated coefficients were significant ($p < 0.05$), except for those for the interaction effect (b_{12}). As combined with the additional results, the standard errors of the coefficients were mostly reduced, and no significant changes ($p > 0.05$) in the estimates of the coefficients occurred. Thus, the repeatability of the optimisation experiments was confirmed, and the refined estimates of the regression model coefficients were used to construct response surfaces shown in Figures 7.1 and 7.2.

A single peak point that represents an optimum glucose and peptone concentrations for the peroxidase production can be seen in Figures 7.1 and 7.2. The optimum glucose and peptone concentrations were determined using the model for each strain and are shown in Table 7.8. Predicted responses and the 95% prediction intervals under the optimum growth conditions were also calculated and are shown in Table 7.8. In order to show the practicality of the models, the predicted responses are also presented in Tables 7.3 and 7.4 along with the experimental data.

Table 7.8. Calculated optimum concentrations of glucose and peptone for the production of peroxidase by two *Coprinus* species, and the responses derived from the models and the confirmation growth experiments.

	Optimum Concentration (%)		Response (U mL^{-1})	
Strain	Glucose	Peptone	Model	Experiment
UAMH 4103	2.9	1.4	70.6 ± 8.3	68.0 ± 3.1
UAMH 10067	2.7	0.8	31.0 ± 6.2	34.5 ± 1.5

Note: The 95% prediction intervals and standard deviations are shown for modelled responses and experimental responses, respectively.

Using the optimised growth conditions shown in Table 7.8, the time series of the growth and peroxidase production of two *Coprinus* species, *Coprinus* sp. UAMH 10067

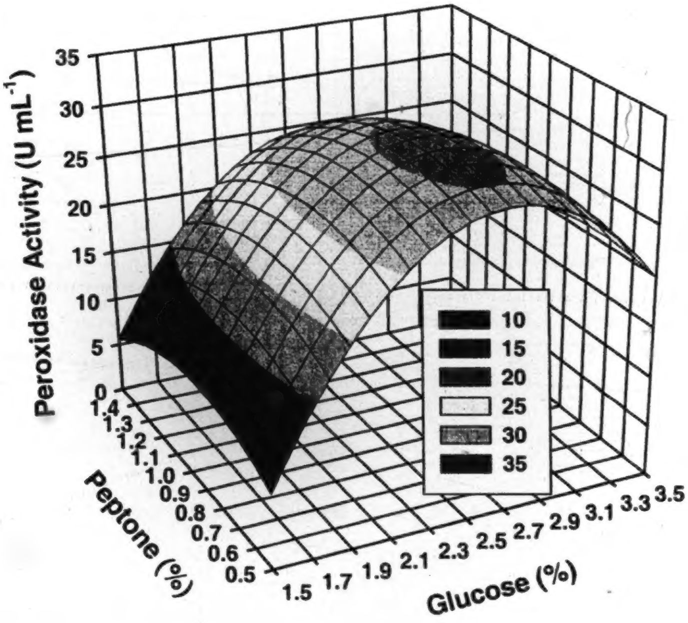


Figure 7.1. Response surface of the central composite design experiment for the peroxidase production by *Coprinus* sp. UAMH 10067.

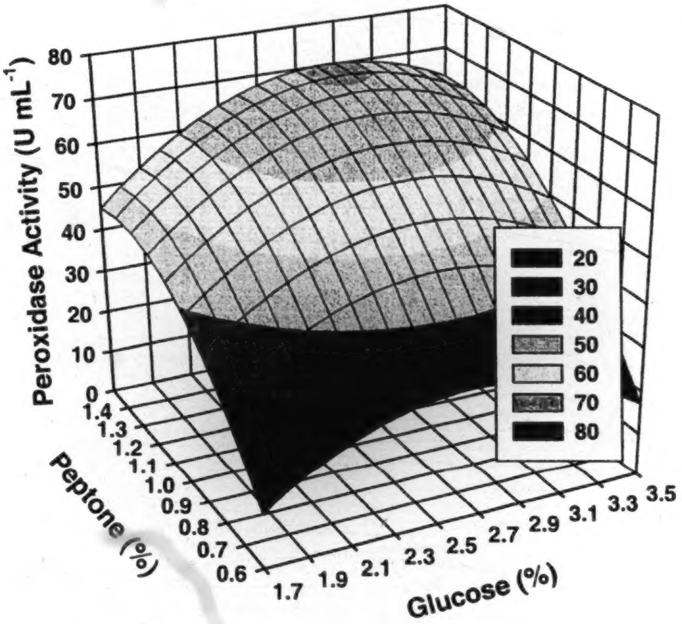


Figure 7.2. Response surface of the central composite design experiment for peroxidase production by *C. cinereus* UAMH 4103.

and *C. cinereus* UAMH 4103, was investigated. The results are shown in Figures 7.3 and 7.4, respectively. Peroxidase activity was observed on day 5 and increased rapidly thereafter in both cases. Peak enzyme activities, 34.5 ± 1.5 U mL^{-1} and 68.0 ± 3.1 U mL^{-1}, were observed on day 14 and on day 12, on which the medium glucose were depleted completely, in the cultures of *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103, respectively. The observed peak peroxidase activities produced by these fungi were more than twice as high as those observed when the original growth medium was used in the previous studies (Ikehata and Buchanan, 2002; Ikehata *et al.*, 2003b). These observed peak peroxidase activities agreed well with the predicted values shown in Table 7.8.

7.4. Discussion

As shown in Table 7.1, among the 8 types of carbon sources, only glucose and fructose supported high production of extracellular peroxidase by *Coprinus* sp. UAMH 10067. While glucose and fructose enter the central metabolic routes of the fungus directly, all other sugars require the activity of several enzymes before entry. The activity of these enzymes is widely rate limiting. Thus, it can be concluded that these monosaccharides are generally better to support the growth and peroxidase production by *Coprinus* species, and there are some preferences within monosaccharides as a carbon source by this fungus.

Of the nitrogen sources examined in this study, complex nitrogen sources, such as peptone and casitone, were generally better than simple inorganic and organic nitrogenous compounds to support the peroxidase production by *Coprinus* sp. UAMH 10067. These complex nitrogen sources contain more readily available organic nitrogen

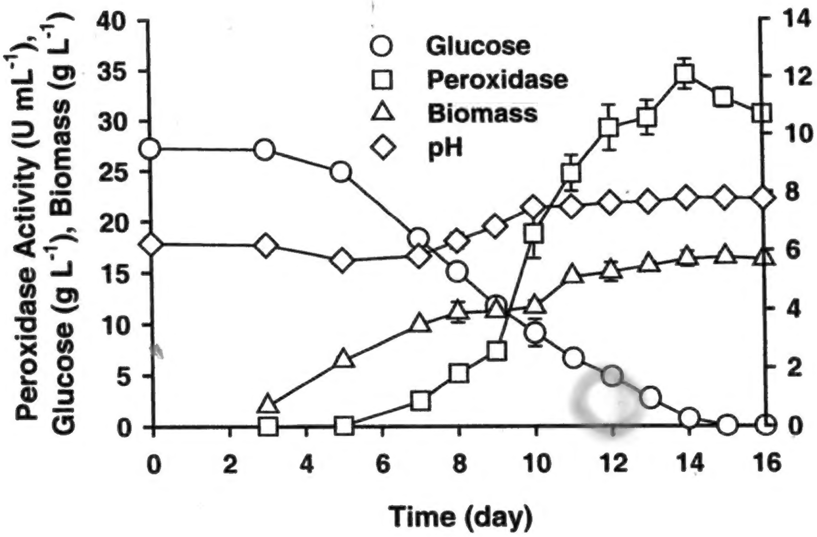


Figure 7.3. Growth of *Coprinus* sp. UAMH 10067 in the optimised medium and its peroxidase production.

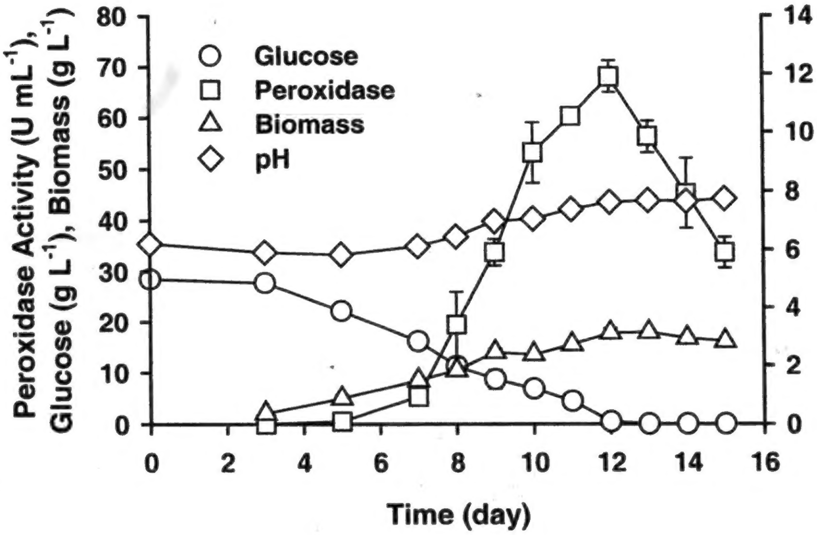


Figure 7.4. Growth of *C. cinereus* UAMH 4103 in the optimised medium and its peroxidase production.

compounds such as amino acids and oligopeptides, as well as minerals and vitamins, which are known to enhance the production of some microbial secondary metabolites (Stanbury *et al.*, 1994).

The result of the screening experiment demonstrated that only two factors, concentration of glucose and concentration of a nitrogen source, were significant for the peroxidase production by *Coprinus* sp. UAMH 10067 (Table 7.6). Since increasing the concentrations of malt extract or yeast extract did not give any significant increase or decrease in peroxidase production, it can be suggested that the components in these compounds (carbohydrates such as maltose and other materials in malt extract, and amino acids, vitamins and minerals in yeast extract) have no effect on peroxidase production within the concentration ranges tested. It was speculated that the addition of ferrous sulphate might promote the peroxidase production because peroxidase is a haemoprotein; however, no effect was observed. This indicates that iron was not limiting in the growth medium.

Medium pH is generally regarded as an important parameter for fermentation processes (Stanbury *et al.*, 1994). However, no significant effect of medium pH was observed on the peroxidase production by *Coprinus* sp. UAMH 10067 within the range tested in the present study. This may be due to the fact that the pH range examined in this study was relatively narrow (pH 5.5 to 6.5). Besides, this range of pH agrees with the medium pH at which the *Coprinus* species grows rapidly and starts producing peroxidase (Figure 7.3). Thus, the medium pH need not be modified if the medium pH value is around 6.

Although glucose concentration showed a major positive effect on the peroxidase production by *Coprinus* sp. UAMH 10067 in the screening experiment, higher glucose concentration showed an inhibitory effect on the growth and the peroxidase production as shown in the result of optimisation experiments (Figure 7.1). Similar effect of peptone concentration on the peroxidase production was also observed in the screening and optimisation experiments. Thus, a fed-batch process would be recommended in the future production study in order to avoid the inhibitory effect at high glucose and peptone concentrations in the growth medium.

The behaviour of another *Coprinus* species, *C. cinereus* UAMH 4103, in the optimisation experiments was very similar (Figure 7.2). However, the optimum growth conditions for peroxidase production were found to be different: while the optimum glucose concentrations were very similar (2.7% for UAMH 10067 and 2.9% for UAMH 4103), the optimum peptone concentrations were substantially different (0.8% and 1.4%, respectively). This fact implies that although in the qualitative part the results of the present study is likely relevant to other peroxidase producing *Coprinus* species, similar optimisation experiments would be needed to determine the optimum culture conditions for each strain of the fungi.

7.5. Conclusion

Optimised culture conditions for peroxidase production by two *Coprinus* species, including *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103, were successfully explored in this study. As a carbon source in the growth medium, glucose and fructose supported the highest level of peroxidase production by *Coprinus* sp. UAMH 10067. It was also shown that complex nutrient sources, including peptone and casitone, were good sources of nitrogen for the peroxidase production. Among the seven factors evaluated in the screening experiment using a 2^{7-3}_{IV} fractional factorial design, glucose concentration and nitrogen source (peptone or casitone) concentration showed significant effects on the peroxidase production. The optimum glucose and peptone concentrations were determined by the subsequent optimisation experiments using central composite designs and regression analyses as 2.7% and 0.8% for *Coprinus* sp. UAMH 10067, and 2.9% and 1.4% for *C. cinereus* UAMH 4103, respectively. Using these optimum culture conditions, the maximum peroxidase activities were observed in the confirmation growth experiments as 34.5 ± 1.5 U mL^{-1} for *Coprinus* sp. UAMH 10067 and 68.0 ± 3.1 U mL^{-1} for *C. cinereus* UAMH 4103, which are more than a 2-fold increase as compared to the results obtained in the previous studies using an original growth medium.

References

Al-Kassim, L., Taylor, K. E., Nicell, J. A., Bewtra, J. K. and Biswas, N. (1994). Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxide from *Coprinus macrorhizus* in batch reactors. *Journal of Chemical Technology and Biotechnology* **61**: 179-182.

Berthouex, P. M. and Brown, L. C. (2002). *Statistics for Environmental Engineers*. Boca Raton, FL, Lewis Publishers.

Box, G. E. P., Hunter, W. G. and Hunter, J. S. (1978). *Statistics for Experimenters - An Introduction to Design, Data Analysis, and Model Building.* New York, NY, John Wiley & Sons, Inc.

Ikehata, K. and Buchanan, I. D. (2002). Screening of *Coprinus* species for the production of extracellular peroxidase and evaluation of the enzyme for the treatment of aqueous phenol. *Environmental Technology* **23**(12): 1355-1368.

Ikehata, K., Buchanan, I. D. and Smith, D. W. (2003a). Treatment of oil refinery wastewater using crude *Coprinus cinereus* peroxidase and hydrogen peroxide. *Journal of Environmental Engineering and Science* (Accepted).

Ikehata, K., Buchanan, I. D. and Smith, D. W. (2003b). Extracellular peroxidase production by *Coprinus* species from urea treated soil. *Canadian Journal of Microbiology* (Submitted).

Kauffmann, C., Petersen, B. R. and Bjerrum, M. J. (1999). Enzymatic removal of phenols from aqueous solutions by *Coprinus cinereus* peroxidase and hydrogen peroxide. *Journal of Biotechnology* **73**: 71-74.

Masuda, M., Sakurai, A. and Sakakibara, M. (2001). Effect of reaction conditions on phenol removal by polymerization and precipitation using *Coprinus cinereus* peroxidase. *Enzyme and Microbial Technology* **28**: 295-300.

Morita, Y., Yamashita, H., Mikami, B., Iwamoto, H., Aibara, S., Terada, M. and Minami, J. (1988). Purification, crystallization, and characterization of peroxidase from *Coprinus cinereus*. *Journal of Biochemistry* **103**(4): 693-699.

Nakayama, T. and Amachi, T. (1999). Fungal peroxidase: its structure, function, and application. *Journal of Molecular Catalysis B-Enzymatic* **6**: 185-198.

Sakurai, A., Kawamoto, S., Abarca, J. F. and Sakakibara, M. (2002). Peroxidase production by *Coprinus cinereus* using rotating disk contactor. *Biochemical Engineering Journal* **10**: 47-53.

Stanbury, P. F., Whitaker, A. and Hall, S. J. (1994). *Principles of Fermentation Technology*. Oxford, U.K., Butterworth Heinemann.

Tsujimura, H., Takaya, M., Katano, K., Matsumoto, N., Park, Y. S. and Okabe, M. (1994).

Scaleup of peroxidase production by *Arthromyces ramosus* based on analysis of fluid velocity distribution. *Journal of Fermentation and Bioengineering* **77**(6): 650-654.

Yao, P.-X., Matsumoto, N., Tsujimura, H., Takaya, M. and Toda, K. (1995). Enhanced production of peroxidase in continuous culture of *Arthromyces ramosus* by selective bleeding of mycelium. *Journal of Fermentation and Bioengineering* **79**(4): 307-312.

Chapter 8. Treatment of Oil Refinery Wastewater Using Crude *Coprinus cinereus* Peroxidase and Hydrogen Peroxide[*]

8.1. Introduction

Many industries generate phenolic pollutants during their manufacturing processes, which has triggered concerns due to their potential health and environmental impacts. In oil refining processes, a major source of phenolic pollutants is the catalytic cracking of a heavy portion of crude oil to produce lighter petroleum products using a catalyst such as zeolites under high temperature and high pressure (US EPA, 1995). Phenols generated during the cracking process are removed from the petroleum products by the addition of inorganic salts such as sodium chloride and calcium chloride along with water and other impurities, and are disposed of with or without treatment (US EPA, 1995). However, the regulations for discharging potentially hazardous phenols are becoming increasingly stringent. Although conventional treatment processes such as biological treatment and chemical oxidation are effective for the removal of phenols from the wastewater, there are several drawbacks, such as high capital and operational costs, low selectivity and low efficiency of biological processes due to the presence of toxic chemicals in the industrial wastewater (Aitken, 1993). Enzymatic treatment using peroxidase and hydrogen peroxide was proposed in the early 1980's as an alternative treatment, which is highly selective and efficient at removing phenols from their aqueous solutions (Klibanov *et al.*, 1980).

---

[*] A version of this chapter has been accepted for publication in July 2003. (Ikehata, K., Buchanan, I.D. and Smith, D.W. *Journal of Environmental Engineering and Science*)

Peroxidase (donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7) catalyses the dehydrogenation of a number of phenols and aromatic amines in the presence of hydrogen peroxide. Hydrogen peroxide oxidises peroxidase to a catalytically active form called Compound I, which is capable of oxidising one molecule of aromatic substrate to a free radical. Compound I is thereby reduced to Compound II, a second catalytically active form, that can oxidise a second phenolic substrate to generate another free radical and return the enzyme to its resting state. Subsequently, these free radicals couple spontaneously to form water-insoluble polymers that can be removed by gravity separation or filtration (Klibanov *et al.*, 1980). A great deal of research has been carried out in the past two decades to investigate the enzymatic treatment of both synthetic and real phenolic wastewaters using peroxidases derived from a variety of sources, including horseradish peroxidase (HRP; Klibanov *et al.*, 1980; Nicell *et al.*, 1993; Wu *et al.*, 1997; Buchanan *et al.*, 1998; Zhang and Nicell, 2000; Wagner and Nicell, 2001, among others), soybean peroxidase (SBP; Caza *et al.*, 1999; Wright and Nicell, 1999; Kinsley and Nicell, 2000), *Coprinus cinereus* (= *C. macrorhizus*) peroxidase (CIP; Al-Kassim *et al.*, 1994a; 1994b; Kauffmann *et al.*, 1999; Masuda *et al.*, 2001a; Ikehata and Buchanan, 2002), and *Arthromyces ramosus* peroxidase (ARP; Buchanan and Han, 2000; Ibrahim *et al.*, 2001; Villalobos and Buchanan, 2002).

Although HRP is the best-studied peroxidase for the treatment of phenolic wastewaters, its production is limited because it is harvested from horseradish roots, which are cultivated mainly for the food industry, grow slowly and require large areas to grow. Since HRP is obtained from the homogenised horseradish roots by means of extraction, the production process is energy and cost intensive and generates a large volume of solid waste. Thus, other peroxidases, such as SBP, CIP and ARP, have drawn much attention as more economically feasible enzymes for the phenolic wastewater treatment during the recent years. Among them, extracellular fungal peroxidases such as ARP and CIP are considered to be suitable for large-scale production because the fungi grow quickly in a small bioreactor and excrete the enzyme to the culture medium in large quantities so that its recovery is less energy and labour intensive.

It was recently reported that unpurified CIP performed better than its purified form in the treatment of synthetic phenolic wastewater (Masuda *et al.*, 2001a; Ikehata and Buchanan, 2002). It has been shown that in the presence of soluble residues of fungal cultures, the enzyme was protected from inactivation caused by the products arising from catalytic oxidation of phenol in synthetic wastewater (Masuda *et al.*, 2001b). The performance of CIP was further improved in synthetic wastewaters by the addition of high molecular weight poly(ethylene glycol) (PEG; Ikehata and Buchanan, 2002), which is a well-known protective agent for other plant and fungal peroxidases (Nakamoto and Machida, 1992; Wu *et al.*, 1997; Buchanan and Han, 2000; Kinsley and Nicell, 2000). However, it had not been demonstrated that these protective effects are also relevant to the treatment of real wastewaters using crude fungal peroxidase, although the protective effect of PEG was observed where crude horseradish extract was used for the treatment of foundry wastewater (Cooper and Nicell, 1996). Additionally, the fate of the soluble fungal residue in crude fungal peroxidase in the wastewater after the enzymatic treatment is not known. This is particularly important because despite its positive effects on the enzymatic treatment, the organic residue in the crude enzyme solution may contribute an additional organic load, which may in turn cause negative impacts on downstream treatment processes as well as on the environment.

Therefore, the objectives of this study were to (1) investigate the treatment of an oil refinery wastewater using crude CIP and hydrogen peroxide; (2) examine the effects of residual organic compounds in the crude enzyme as well as the addition of high molecular weight PEG on the catalytic transformation of phenols in the wastewater, and on the organic content of the treated wastewater; and (3) compare the performance of CIP to those of purified HRP and ARP in the treatment of a strong refinery wastewater. Furthermore, since preliminary results showed that the peroxidase-mediated treatment of the oil refinery wastewater did not produce precipitate, but coloured colloidal products, the formation of the coloured products and their removal was also studied.

8.2. Materials and methods

8.2.1. Materials

8.2.1.1. Enzymes

Crude CIP was prepared by liquid fermentation of *C. cinereus* UAMH 4103 (obtained from the University of Alberta Microfungus Collection and Herbarium, Alberta, Canada) as described previously (Ikehata and Buchanan, 2002). Biomass-free fungal culture supernatant was used as a crude stock of CIP without further purification. The peroxidase activity in the crude CIP stock solution was approximately 25 U mL^{-1}, based on the phenol/4-AAP assay described herein. Peroxidases from horseradish (HRP; RZ = 1.1) and from *Arthromyces ramosus* (ARP; RZ = 2.5) and catalase (EC 1.11.1.6) from bovine liver were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

8.2.1.2. Chemicals

99% 3,5-dichloro-2-hydroxybenzenesulphonic acid sodium salt (HDCBS; Aldrich brand), ferric chloride hexahydrate, 50 wt.% aqueous solution of polyacrylamide (average M.W. = 10 kDa; Aldrich brand), 20 wt.% aqueous solution of poly(diallyldimethylammonium chloride) (poly(DADMAC); average M.W. = 400 to 500 kDa; Aldrich brand) and PEG (average M.W. = 35 kDa; Fluka brand) were purchased from Sigma-Aldrich Canada Ltd.. Ammonium chloride, aluminum sulphate (alum), 98% 4-aminoantipyrine (4-AAP), calcium chloride, glutamic acid, 30% w/w hydrogen peroxide, magnesium sulphate heptahydrate, mercuric sulphate, phenol, Polyseed for BOD, potassium dichromate, potassium ferricyanide, potassium hydrogen phthalate, potassium phosphate monobasic, silver sulphate, sodium bicarbonate, sodium hydroxide, sodium phosphate monobasic, sodium phosphate dibasic and sulphuric acid (all ACS certified except for 4-AAP and Polyseed) were purchased from Fisher Scientific Canada. Ultrapure water produced by Elgastat Maxima Water Purification System (Elga Ltd., High Wycombe, Bucks, England) was used for all reagent preparation.

8.2.1.3. Oil refinery wastewater

A wastewater sample was obtained from a drier unit of the catalytic treatment process of an oil refinery, because it contained high level of phenols. Chemically treated light catalytic cracked distillates (LCCD) containing hydrocarbons having carbon numbers predominantly in the range of C9 through C25 were passed through solid sodium chloride/calcium chloride in the driers, in which moisture in the LCCD was removed as wastewater along with phenols generated during catalytic cracking, trace LCCD, sodium and calcium chloride and other impurities. The sample was stored at 4°C upon arrival. The wastewater characteristics determined in the laboratory are summarised in Table 8.1.

Table 8.1. Characteristics of oil refinery wastewater.

pH	Total phenol (mM)	$Abs_{410\ nm}$* ($OD^{\dagger}$)	COD (mg L^{-1})	BOD_5 (mg L^{-1})	Cl^- (g L^{-1})
11.4	64.5	0.65	25.9×10^3	10.0×10^3	1.1

Note:

* absorbance at 410 nm

† optical density

8.2.2. Equipment

A Hewlett-Packard HP 8453 UV-Visible Spectrophotometer was used for the measurement of UV and visible spectra of samples, and colourimetric assays for peroxidase activity and concentrations of hydrogen peroxide and phenol. An Ultrospec 2000 UV-Visible Spectrometer (Pharmacia Biotech Ltd., Cambridge, England) was used for COD test. Glass and quartz semi-micro cuvettes with a 10 mm optical path and a 1.5 mL volume (Hellma Ltd., Canada) were used in all spectrophotometric measurements. A Centra-GP8R Refrigerated Centrifuge (International Equipment Company, Needham Heights, Massachusetts) was used for solid-liquid separation of samples.

8.2.3. Analytical methods

8.2.3.1. Peroxidase activity assay

Peroxidase activity was measured by a colourimetric assay in which 1 mL assay mixture was composed of 500 μL of 20 mM phenol, 250 μL of 9.6 mM 4-AAP, 100 μL of 2 mM hydrogen peroxide, 0 to 130 μL of 0.1 M sodium phosphate buffer at pH 7.4 and 20 to 150 μL of sample. All assay reagents were prepared in the 0.1 M sodium phosphate buffer at pH 7.4. Immediately after the addition of sample, the formation of quinoneimine dye was monitored using a spectrophotometer at a wavelength of 510 nm at 25°C. One unit (U) of peroxidase activity was defined as the amount of hydrogen peroxide consumed (in μmole) in one minute using an extinction coefficient of 7 100 M^{-1} cm^{-1} based on hydrogen peroxide.

8.2.3.2. Hydrogen peroxide assay

Hydrogen peroxide concentration was measured by a colourimetric assay at 25°C in which 1 mL assay mixture was composed of 500 μL of 18 mM HDCBS, 250 μL of 9.6 mM 4-AAP, 100 μL of 1 mg mL^{-1} HRP solution, 0 to 130 μL of 0.1 M sodium phosphate buffer at pH 7.4 and 20 to 150 μL of sample. All reagents were prepared in 0.1 M sodium phosphate buffer at pH 7.4. The colour was allowed to develop for 8 minutes after the reagents were combined and was measured using a spectrophotometer at a wavelength of 510 nm. This absorbance was converted to hydrogen peroxide concentration using a calibration curve.

8.2.3.3. Total phenol assay

Total phenol concentration in oil refinery wastewater was measured by a colourimetric assay in Section 5520 of Standard Methods (APHA *et al.*, 1995) with modification to handle samples with higher phenol content. The phenolic compounds in a sample react with 4-AAP in the presence of potassium ferricyanide at 25°C. An aliquot of sample was diluted and brought to a volume of 800 μL with 0.25 M sodium bicarbonate. Subsequently, 100 μL of 20.8 mM 4-AAP followed by 100 μL of 83.4 mM potassium ferricyanide (both prepared in 0.25 M sodium bicarbonate) were added to the sample. The absorbance of the assay mixture at 510 nm was measured after 6 minutes. When a sample had an absorbance at 510 nm prior to the assay, this absorbance was subtracted from the absorbance measured during the assay. The absorbance at 510 nm due to the reaction between phenol compounds and assay reagents was subsequently converted to a total phenol concentration with a calibration curve using phenol (C_6H_5OH) as a standard. It should be noted that the total phenol assay was not applicable to para-substituted phenols (APHA *et al.*, 1995). Thus, the total phenol concentration determined by this method may have been underestimated when substantial amounts of para-substituted phenols were present in the sample.

8.2.3.4. Chemical and biochemical oxygen demands and colour measurement

Chemical and 5-day biochemical oxygen demands (COD and BOD_5) were determined according to the sections 5220 and 5210 of Standard Methods (APHA *et al.*, 1995), respectively. Polyseed (Fisher Scientific) in aerated BOD dilution buffer was used as a seed for the BOD_5 test. Absorbance at 410 nm of wastewater samples was measured with a spectrophotometer before and after the treatment because this was the wavelength at which the coloured products of enzymatic treatment exhibited peak absorbance as discussed below. The enzymatically treated wastewater samples were diluted as required prior to the absorbance measurement to ensure that the absorbance at 410 nm of resultant solution was less than 1.

8.2.4. Experimental procedures

8.2.4.1. Batch treatment experiments

A wastewater sample was first diluted with pure water to 50% (v/v) and adjusted to a desired pH with 12 *N* sulphuric acid. In either 30 mL or 100 mL borosilicate vials, an aliquot of hydrogen peroxide and 2 g L^{-1} PEG aqueous solution, if necessary, were added to the wastewater and further diluted to the desired total phenol concentration with an adequate amount of ultrapure water. Enzymatic reaction was initiated by the addition of a known amount of stock peroxidase solution. Reactor contents were stirred with a Teflon coated stir bar and a magnetic stirrer. All vials were covered with lids to avoid volatilisation. After 2 hours of incubation at room temperature (18 ± 1°C), the treated wastewater was coagulated with 400 mg L^{-1} of alum and centrifuged at 2 000 *g* for 30 minutes to remove suspended solids. No pH adjustment was made prior to the alum coagulation because pH of the coagulated wastewater never deviated from the effective pH range for alum coagulation (pH 4.5 to 7). The supernatant was analysed for residual concentrations of total phenol and hydrogen peroxide, peroxidase activity, COD, BOD_5 and colour. Before preparing the BOD bottles, residual hydrogen peroxide was completely destroyed with 0.03 g L^{-1} catalase to avoid it decomposing during the BOD incubation period and contributing to the dissolved oxygen in the bottles. All treatments were performed at least twice.

8.2.4.2. Colour and product removal experiments

In a 600 mL beaker, the diluted and neutralised wastewater was combined with hydrogen peroxide and water to a desired total phenol concentration. In order to initiate the enzymatic reaction, a proper amount of crude CIP solution was added to the wastewater, stirred with a Teflon coated stir bar and a magnetic stirrer. After 2 hours of reaction at room temperature (18 ± 2°C), the treated wastewater was divided into 30 mL borosilicate vials (8 mL of the sample each), in which "jar tests" for the removal of coloured products was performed with various doses of alum ranging from 25 mg L^{-1} to 600 mg L^{-1}. The effect of the addition of either of two polyelectrolytes, polyacrylamide or poly(DADMAC), as a coagulant aid was also examined. The final concentration of either polyelectrolyte in the reaction mixtures ranged from 1 mg L^{-1} to 10 mg L^{-1}. Various amounts of alum and concentrated polyelectrolyte solution were added at the same time to samples stirred with a magnetic stirrer. After an intense mixing for 30 seconds and following flocculation for one minute, the samples were transferred to test tubes and centrifuged at 2 000 *g* for 30 minutes to remove solid precipitates. The absorbance of the sample at 410 nm, at which the soluble reaction products absorb visible light, was recorded as a measure of residual colour and reaction products. All treatments were performed at least twice.

8.3. Results and discussion

Since the complete treatment of the full-strength oil refinery wastewater would have required large volumes of crude CIP solution, the wastewater was diluted with pure water. A 10-fold dilution that gives rise to a wastewater containing 6.4 mM total phenol was used in most cases. The enzymatic treatment of the diluted oil refinery wastewater with peroxidase did not result in the formation of solid precipitates, but produced dark-coloured suspended products that could not be removed by centrifugation at 2 000 *g* for 30 minutes. However, in cases where more concentrated wastewater containing 9.6 mM total phenol was treated, small amounts of black, oily substance were obtained after the centrifugation, while most of the coloured products remained in the solution. Since the coloured products interfered with the colourimetric total phenol assay, they were removed prior to the phenol measurements by coagulation with 400 mg L^{-1} of alum and subsequent centrifugation at 2 000 *g* for 30 minutes.

8.3.1. Effect of pH

The effect of reaction pH on the CIP-mediated treatment of the oil refinery wastewater was examined with a limited amount of enzyme at various pHs. The results are shown in Figure 8.1. Since the wastewater had little buffering capacity, the pH of the reaction mixture was changed markedly after the addition of enzyme because the crude CIP solution had an initial pH of 7.3. Therefore, the wastewater pH after the enzyme addition was recorded and plotted as the ordinates of Figure 8.1.

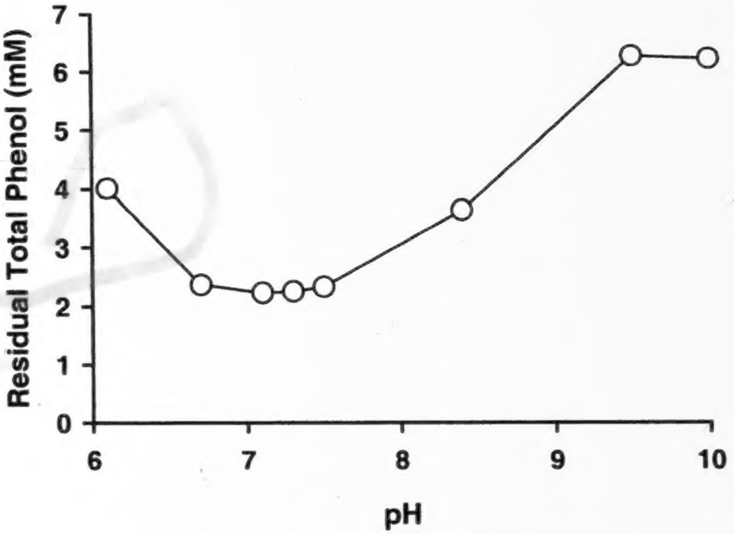


Figure 8.1. Residual total phenol in the oil refinery wastewater as a function of reaction pH after 2 hours of reaction and subsequent coagulation with 400 mg L^{-1} of alum at 18 ± 1°C ($[\text{Total Phenol}]_0 = 6.4$ mM, $[H_2O_2]_0 = 10$ mM, $[\text{CIP}]_0 = 1.5$ U mL^{-1}).

No reduction in the total phenol was observed in control vials from which either enzyme or hydrogen peroxide was absent. Nearly constant total phenol transformations were observed from pH 6.6 to 7.4. Outside this range, the transformation efficiency, which can be defined as the transformed total phenol divided by initial total phenol concentration, was lowered. Based on the results shown in Figure 8.1, sample wastewater was adjusted to pH 7.0 for all subsequent tests. This pH increased to 7.2 after CIP addition and remained virtually constant during the treatment.

8.3.2. Effect of hydrogen peroxide concentration

The effect of initial hydrogen peroxide concentration on the CIP-mediated treatment of the oil refinery wastewater containing 6.4 mM of total phenol was examined with 4.3 U mL^{-1} enzyme and various initial concentrations of hydrogen peroxide. The results are shown in Figure 8.2. Residual total phenol decreased with increasing amounts of initial hydrogen peroxide provided, and all total phenol in the wastewater was completely transformed when an 8 mM initial concentration of hydrogen peroxide was provided. Some spontaneous decomposition of hydrogen peroxide (1.1 mM decrease) was observed in control vials, which initially contained 10 mM H_2O_2 and wastewater but no CIP, without affecting total phenol concentration. This is likely due to the presence of reduced sulphur compounds, such as sulphide, in the refinery wastewater, which can be oxidised by hydrogen peroxide to sulphur (Wagner and Nicell, 2002).

The apparent stoichiometry between hydrogen peroxide consumed and total phenol transformed was calculated to be 1.2 based on the results presented in Figure 8.2. This was slightly higher than that observed in a synthetic wastewater system where it was 1.0 (Ikehata and Buchanan, 2002). There are at least two causes of this phenomenon: 1) the possible underestimation of total phenol concentration by the colourimetric method used in this study; and 2) the presence of reducing agents, such as sulphide. In addition to the non-enzymatic oxidation of sulphide by hydrogen peroxide, sulphide in refinery wastewater may reduce phenoxy radicals back to phenols and increase hydrogen peroxide demand (Wagner and Nicell, 2002).

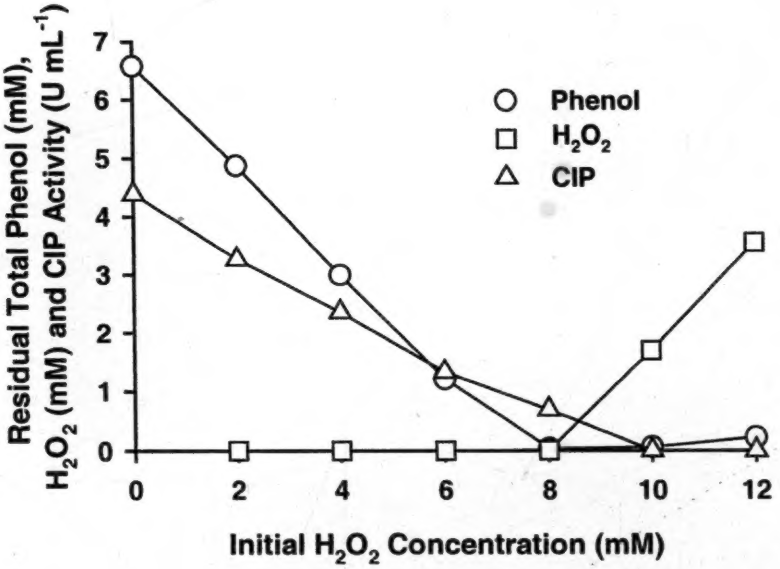


Figure 8.2. Effect of initial hydrogen peroxide concentration on residual total phenol, hydrogen peroxide and CIP activity in the oil refinery wastewater after 2 hours of reaction and subsequent coagulation with 400 mg L^{-1} of alum at 18 ± 1°C ([Total Phenol]$_0$ = 6.4 mM, [CIP]$_0$ = 4.3 U mL^{-1}).

As shown in Figure 8.2, CIP was inactivated during the catalytic transformation of phenolic compounds in oil refinery wastewater. No residual CIP activity was detected where excess hydrogen peroxide was provided. Since no marked loss in CIP activity was observed over the 2-hour reaction period in the wastewater in the absence of hydrogen peroxide, it is likely that the CIP was inactivated by the excess hydrogen peroxide present in the reaction mixtures. This fact suggests that it is necessary to optimise the amount of hydrogen peroxide in order to save the amount of CIP required for refinery wastewater treatment.

8.3.3. Effects of peroxidase concentration, type and purity of peroxidase, and PEG addition

The effect of initial CIP concentration on the phenol transformation in the oil refinery wastewater was examined alternatively in the presence and in the absence of PEG. Additionally, two purified peroxidases, ARP and HRP, were also examined to study the effect of enzyme type and purity on the phenol transformation. ARP was chosen as an alternative of the pure CIP because these two fungal enzymes are considered identical (Sawai-Hatanaka *et al.*, 1995), and purified CIP is not commercially available. HRP was chosen because it is the most studied plant peroxidase for phenolic wastewater treatment. The concentrations of initial total phenol, hydrogen peroxide and PEG were fixed at 6.4 mM, 10 mM, and 200 mg/L (if added), respectively. The results are summarised in Figure 8.3.

Although all three peroxidases catalysed the transformation of phenolic compounds in the refinery wastewater, the relationships between initial peroxidase activity and residual total phenols were quite different between the fungal peroxidases (CIP and ARP) and HRP. While HRP appeared to be less efficient at phenol transformation in the absence of PEG than the fungal peroxidases, the transformation efficiency greatly improved in the presence of PEG. In contrast, the transformation efficiency was essentially the same for both pure ARP and crude CIP, both in the presence and in the absence of PEG. No improvement was observed where either more (400 mg

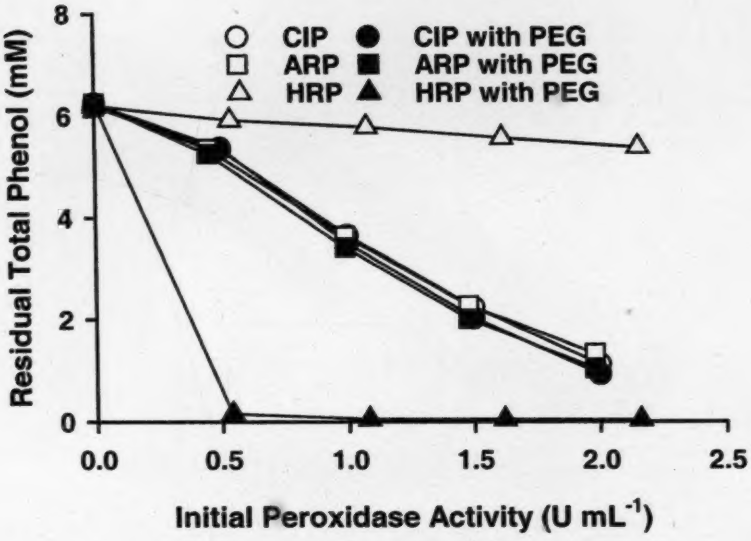


Figure 8.3. Residual total phenol in the oil refinery wastewater, after 2 hours of reaction and subsequent coagulation with 400 mg L^{-1} of alum at 18 ± 1°C, as a function of initial peroxidase activity and the type of enzyme in the presence (closed symbols) and in the absence (open symbols) of 200 mg L^{-1} PEG ([Total Phenol]$_0$ = 6.4 mM, [H_2O_2]$_0$ = 10 mM).

L^{-1}) or less (50 mg L^{-1}) PEG was supplied to the reaction mixture (data not shown).

The observed effect of PEG on the HRP-mediated phenol transformation in the oil refinery wastewater is consistent with the other studies using HRP to treat either synthetic (Nakamoto and Machida, 1992; Wu *et al.*, 1997) or real phenolic wastewaters (Nakamoto and Machida, 1992; Cooper and Nicell, 1996; Wagner and Nicell, 2001). The PEG molecules and the polymeric products of phenol oxidation by peroxidase are thought to interact and reduce the chance of enzyme adsorption, entrapment and inactivation by the polymeric products (Nakamoto and Machida, 1992; Buchanan and Nicell, 1997).

The results of the present study are different in two senses from all other reported studies using fungal peroxidases, including ARP and CIP, for phenolic wastewater treatment. Firstly, there was no improvement in phenol transformation efficiency by the addition of PEG. Secondly, the purity of fungal peroxidases did not have an effect on the phenol transformation. Similar to the cases of other peroxidases, ARP-mediated phenol removal was improved by the addition of PEG in aqueous phenol solution (Ibrahim *et al.*, 1997; Buchanan and Han, 2000) as well as in a weak oil refinery wastewater spiked with phenol (Ibrahim *et al.*, 2001). In addition, crude CIP performed better than purified enzyme in the transformation and subsequent removal of phenol from the synthetic mixture (Masuda *et al.*, 2001a; Ikehata and Buchanan, 2002). It was suggested that the impurities, such as polysaccharides and proteins, derived from fungal culture present in crude CIP were likely to have a protective effect on the peroxidase similar to that of PEG (Masuda *et al.*, 2001b; Ikehata and Buchanan, 2002).

There is no clear explanation of the inconsistent effects of PEG and fungal residues on the catalytic phenol transformation observed in this study. Although the substrate specificities and catalytic activities of fungal peroxidases are known to be very similar to those of HRP (Kjalke *et al.*, 1992; Farhangrazi *et al.*, 1994), the molecular structures and some of the catalytic features of these enzymes are substantially different (Farhangrazi *et al.*, 1994; Sawai-Hatanaka *et al.*, 1995; Tanaka *et al.*, 1999). It can be suggested that the fungal peroxidases and HRP may have different inactivation mechanisms. Further studies will be needed to elucidate this issue.

8.3.4. Impact of enzymatic treatment on the COD and BOD$_5$ of the wastewater

Based on the results reported in the previous section, the use of crude CIP in the oil refinery wastewater treatment is apparently more advantageous than that of the other two enzymes for two reasons: 1) minimal purification of enzyme is needed, which will reduce the enzyme production cost substantially; and 2) PEG addition is not necessary. However, the crude CIP contains large amounts of organic compounds derived from liquid culture residues that may have impacts on the downstream treatment facilities. Therefore, the fate of impurities after the enzymatic treatment was studied using BOD_5 and COD as measures of organic compounds in the wastewater.

Before conducting the treatment of oil refinery wastewater, the COD and BOD_5 of crude and purified fungal peroxidases were determined. The crude CIP used in this study exerted 340 mg COD L^{-1} and 110 mg BOD_5 L^{-1} in the solution containing 1 U mL^{-1} of active enzyme. The aqueous solution of purified ARP containing the same level of active enzyme exerted 10 mg COD L^{-1} and a negligible amount of BOD_5. The initial COD and BOD_5 of the wastewater sample containing 6.4 mM total phenol were 2580 mg L^{-1} and 940 mg L^{-1}, respectively. The hydrogen peroxide concentration was fixed at 10 mM for the enzymatic treatment.

Figure 8.4 shows the results from the tests conducted to assess the effect on the oxygen demands of treatment with CIP or APR. Results are expressed as either before coagulation (BC) or after coagulation (AC). All samples underwent enzymatic treatment, and the BC results were obtained from the samples immediately after this treatment, whereas the AC results are from the samples that had been coagulated with 400 mg L^{-1} alum and centrifuged to remove suspended solids. After the enzymatic treatment but before coagulation, total COD increased slightly in the wastewater where crude CIP was used. COD slightly decreased before coagulation where purified ARP was used. The increase in total COD in the CIP-treated wastewater was likely attributed to the soluble organic residues of fungal culture in the crude CIP. BOD_5, on the other hand, was reduced

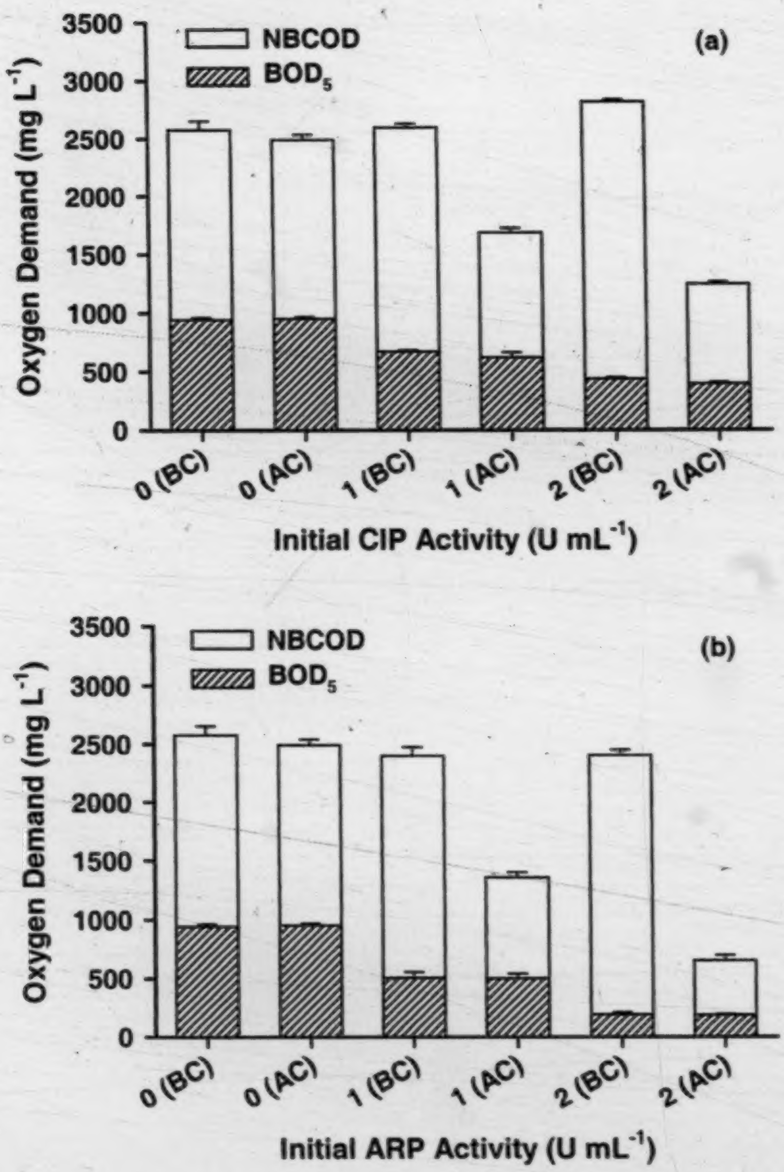


Figure 8.4. COD and BOD_5 of the oil refinery wastewater treated with (a) crude CIP or (b) purified ARP at 18 ± 1°C before and after coagulation with 400 mg L^{-1} of alum (NBCOD: not-readily biodegradable COD (= Total COD – BOD_5), $[\text{Total Phenol}]_0 = 6.4$ mM, $[H_2O_2]_0 = 10$ mM). The "BC" and "AC" notation in the figure represents before and after coagulation, respectively. Error bars represent standard deviation of two treatments.

during the enzymatic treatment proportional to increasing amounts of peroxidase. This implies that some of the readily biodegradable compounds, presumably phenolic compounds, were converted to not-readily-biodegradable substances. This is consistent with the fact that the enzymatic treatment of phenol involves polymerisation of phenoxy radicals to high molecular weight polymers that are expected to be less biodegradable than parent phenolic compounds. This underscores the need to characterise the reaction products arising from peroxidase-catalysed treatment of phenols.

After alum coagulation and centrifugation, some of the not-readily-biodegradable COD (dubbed as NBCOD) was removed from the wastewater, while BOD_5 remained virtually unchanged. Compared to the controls in which no peroxidase was added (0 U mL^{-1}), the amounts of NBCOD that could be removed by coagulation increased with increasing amounts of peroxidase, regardless of the enzyme type. This indicates that some not-readily biodegradable compounds, presumably including some substituted phenols, were removed by the enzymatic treatment and subsequent coagulation. As a result of enzymatic treatment ($[\text{peroxidase}]_0 = 2$ U mL^{-1}) and alum coagulation, the total COD and BOD_5 were reduced by 52% and 58% with crude CIP, and 75% and 80% with purified ARP, respectively.

The wastewater treated with crude CIP always contained more COD and BOD_5 than that treated with purified ARP even after alum coagulation. A comparison of Figure 8.4a to Figure 8.4b shows that the differences in residual COD and BOD_5 between the treatments using CIP and ARP after coagulation (AC) were 598 mg COD L^{-1} and 210 mg BOD_5 L^{-1} when 2 U mL^{-1} enzyme was added. These differences are nearly equal to the differences in oxygen demands between the stock solutions of the two peroxidases. This implies that the fungal residues in crude CIP were removed neither by enzymatic treatment nor by coagulation. Although the fungal residues were mostly biodegradable, the introduction of additional organic compounds would not be desirable for wastewaters in which the initial oxygen demands were small, and no downstream biological treatment process was planned. Therefore, at least partial purification of the enzyme would be recommended to remove some organic residues prior to its introduction into actual treatment processes.

8.3.5. Colour and product removal by coagulation

Although the coloured products formed by enzymatic treatment of oil refinery wastewater were successfully removed by coagulation with 400 mg alum L^{-1} in the results shown previously, the relationships between amount of the coloured products formed and the amount of total phenol transformed and removed, as well as the minimum amount of alum required to remove the products had not yet been established. Therefore, the formation of coloured products and the effect of coagulation on the products were investigated with various concentrations of wastewater, treated with various amounts of CIP. The use of nonionic and anionic polymers as a coagulant aid was also examined. Since the visible spectrum of the wastewater treated with CIP had a broad peak near 410 nm (Figure 8.5), the absorbance at 410 nm was monitored as a measure of the concentration of coloured products. The COD of the treated wastewater was also measured before and after coagulation to determine the actual amount of products removed from the wastewater.

The colour removal experiments were conducted after 2 hours treatment when all enzymatic reactions were completed. As shown in Figure 8.5, the absorbance at 410 nm was removed almost completely by coagulation with an appropriate amount of alum.

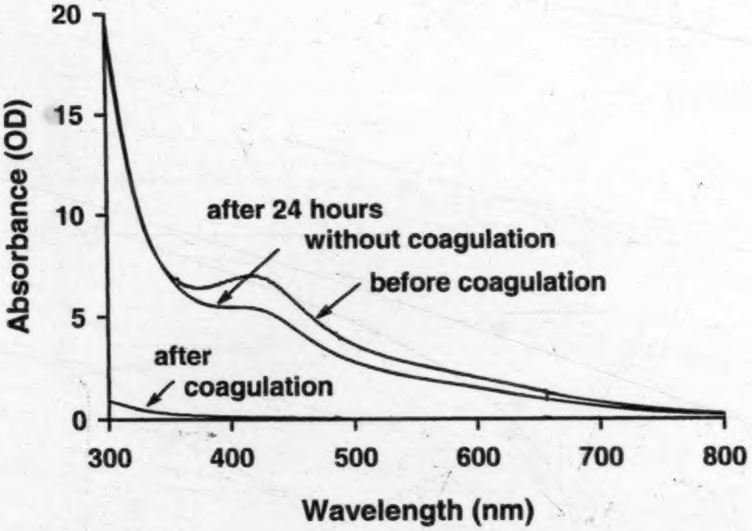


Figure 8.5. Visible spectra of the oil refinery wastewater treated with CIP at 18 ± 1°C before and after coagulation with 400 mg L^{-1} of alum, and after 24 hours without coagulation ([Total Phenol]$_0$ = 6.4 mM, [H_2O_2]$_0$ = 10 mM, [CIP]$_0$ = 3.5 U mL^{-1}).

It should be noted that after 24 hours of incubation in a dark place at room temperature, the absorbance at 410 nm of the treated wastewater reduced to about 73% of the value measured immediately after the enzymatic treatment (Figure 8.5). Because no precipitate was formed, this may imply that some of the products were not stable and transformed or decomposed into other molecules that absorbed less visible light, although no increase in phenol concentration was detected by the total phenol assay in the aged wastewater treated with CIP.

Figure 8.6 shows the relationships between the transformed total phenol and the absorbance at 410 nm generated by enzymatic treatment, as well as the amount of COD removed by coagulation with 400 mg alum L^{-1} from oil refinery wastewater containing 6.4 mM of total phenol treated with various amounts of CIP. There is a linear relationship

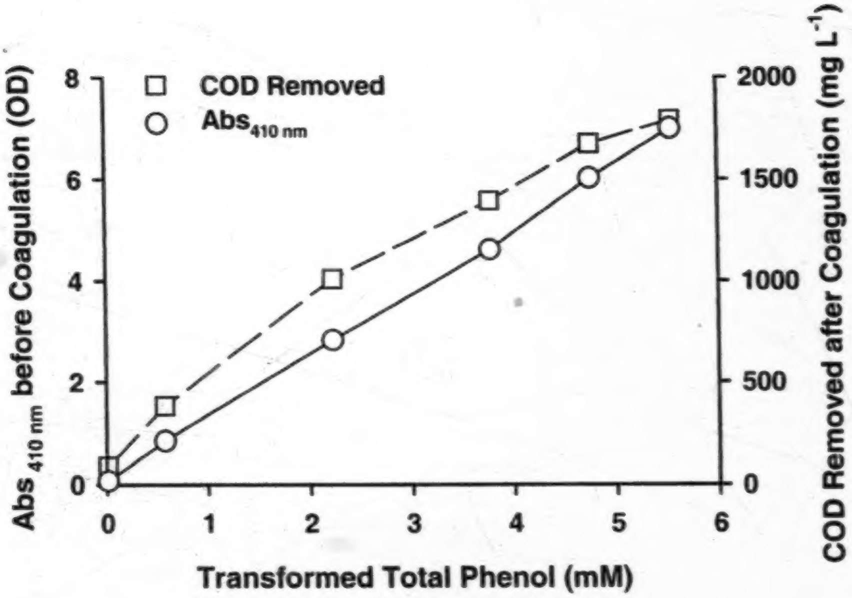


Figure 8.6. Relationships between transformed total phenol and absorbance at 410 nm (Abs$_{410 nm}$) before coagulation as well as COD removed after coagulation in the oil refinery wastewater treated with various amounts of CIP at 18 ± 1°C ([Total Phenol]$_0$ = 6.4 mM, [H_2O_2]$_0$ = 10 mM, [CIP]$_0$ = 0.5 – 2.5 U mL^{-1}, alum dose = 400 mg L^{-1}).

between phenol transformed and absorbance at 410 nm generated by the enzymatic treatment with CIP, which can be expressed as following equation:

$$\text{Abs.}_{@410\text{ nm}}\text{ (OD)} = 1.24 \times \text{Phenol transformed (mM)} + 0.07 \qquad (8\text{-}1)$$

Since there was nearly no residual absorbance at 410 nm after the coagulation with alum, the absorbance at 410 nm removed by coagulation was equal to that generated by the enzymatic treatment. Unlike the absorbance at 410 nm, the amount of COD removed did not show an apparent linear relationship to the transformed total phenol. This might be due to the complexity of the refinery wastewater, in which a variety of phenolic species may be present. These phenolic compounds vary in molecular weight, which would affect COD, as well as in the type, number and position of substituted functional groups. Since these phenolic compounds have various affinities to peroxidase (Aitken and Heck, 1998), they are likely to compete with each other during the enzymatic treatment. On the other hand, since a major chromophore of the polymeric products is considered to be conjugated aromatic rings and may be affected little by the substituted functional groups, the relationship between transformed total phenol and generated colour may be rather straightforward as observed. Nevertheless, since the amounts of absorbance at 410 nm removed after coagulation was closely related to the amounts of COD removed as shown in Figure 8.6, the absorbance at 410 nm was used as an indicator of the residual concentration of polymeric products in the following colour removal experiments.

The effect of alum dose on the removal of coloured products was examined using the wastewaters with three levels of initial total phenol (3.2, 6.4 and 9.6 mM) treated with enough CIP to transform more than 98% of the total phenol. The results are summarised in Figure 8.7. It should be noted that all absorbance data presented in Figure 8.7 were recorded after centrifugation. Although the absorbance at 410 nm in the enzymatically treated wastewater initially containing 9.6 mM total phenol appears to be 7.7, more coloured products were actually present in the wastewater before the jar tests because small amounts of dark coloured, oily substances were removed from the enzymatically treated wastewater by centrifugation with no addition of coagulant, and reduction in the absorbance was observed.

More than 98% of coloured products were removed with 200 mg L^{-1}, 250 mg L^{-1} and 600 mg L^{-1} of alum from the treated wastewaters that originally contained 3.2, 6.4 mM and 9.6 mM of total phenol, respectively. More alum was obviously required to

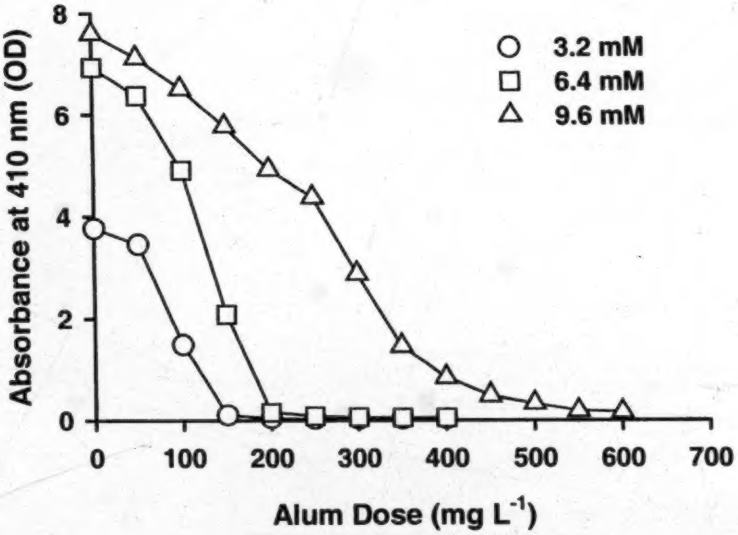


Figure 8.7. Removal of coloured products from the oil refinery wastewater treated with CIP at 18 ± 1°C as a function of alum dose for three initial total phenol concentrations (3.2, 6.4 and 9.6 mM). More than 98% of initial total phenol was transformed ($[H_2O_2]_0 = 5 - 15$ mM, $[CIP]_0 = 2 - 8$ U mL^{-1}).

remove coloured product from stronger wastewater. There was no clear relationship between the initial total phenol concentration and the amounts of alum required to remove 98% of coloured products. This suggests that it is necessary to perform a jar test to decide the amount of alum for each wastewater to be treated. The use of two types of polyelectrolytes as a coagulant aid was tested; however, neither a nonionic polymer, polyacrylamide, nor an anionic polymer, poly(DADMAC), was effective in reducing the amount of alum required (data not shown).

8.4. Conclusions

Enzymatic treatment of oil refinery wastewater was investigated using crude CIP containing residues from liquid fungal culture. Phenols in the wastewater were converted to coloured polymeric products by the catalytic reactions with CIP and hydrogen peroxide. Since the majority of the products could not be removed by centrifugation, coagulation with alum was needed to remove them from the wastewater. The optimum pH for the treatment of oil refinery wastewater with crude CIP was nearly neutral. More hydrogen peroxide was required to transform a given amount of phenol in the real wastewater than in synthetic wastewater. Unlike previously reported studies with synthetic phenolic wastewater, neither the presence of residual fungal residues nor the addition of PEG had an effect on the phenol transformation catalysed by two fungal peroxidases, CIP or ARP, in the oil refinery wastewater, while the PEG addition improved HRP-catalysed phenol transformation. As a result of enzymatic treatment ($[\text{peroxidase}]_0 = 2\ \text{U mL}^{-1}$) and alum coagulation of the wastewater containing 6.4 mM total phenol, the total COD and BOD_5 were reduced by 52% and 58% with crude CIP, and 75% and 80% with purified ARP, respectively. Although COD and BOD_5 were removed from the wastewater by the enzymatic treatment and subsequent coagulation with alum, the dissolved organic materials in the crude CIP were unlikely to have been affected by either process and tended to remain in the treated wastewater. Therefore, partial purification of the crude enzyme prior to its application to wastewater treatment would be recommended where initial oxygen demands in the wastewater were small, and no downstream biological treatment process exists. Although there was a linear relationship between coloured products generated by enzymatic treatment and total phenol transformed in the oil refinery wastewater, there was no clear relationship between the initial total phenol concentration and the amounts of alum required to remove the coloured products generated. Thus, it is recommended that a jar test be performed to decide the amount of alum for each refinery wastewater to be treated.

References

Aitken, M. D. (1993). Waste treatment applications of enzymes: opportunities and obstacles. *The Chemical Engineering Journal* **52**: B49-B58.

Aitken, M. D. and Heck, P. E. (1998). Turnover capacity of *Coprinus cinereus* peroxidase for phenol and monosubstituted phenols. *Biotechnology Progress* **14**(3): 487-492.

Al-Kassim, L., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1994a). Optimization of phenol removal by a fungal peroxidase from *Coprinus macrorhizus* using batch, continuous, and discontinuous semibatch reactors. *Enzyme and Microbial Technology* **16**: 120-124.

Al-Kassim, L., Taylor, K. E., Nicell, J. A., Bewtra, J. K. and Biswas, N. (1994b). Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxide from *Coprinus macrorhizus* in batch reactors. *Journal of Chemical Technology and Biotechnology* **61**: 179-182.

APHA, AWWA and WEF (1995). *Standard Methods*. Washington, DC, American Public Health Association.

Buchanan, I. D. and Han, Y. S. (2000). Assessment of the potential of *Arthromyces ramosus* peroxidase to remove phenol from industrial wastewaters. *Environmental Technology* **21**: 545-552.

Buchanan, I. D. and Nicell, J. A. (1997). Model development for horseradish peroxidase catalyzed removal of aqueous phenol. *Biotechnology and Bioengineering* **54**(3):

251-261.

Buchanan, I. D., Nicell, J. A. and Wagner, M. (1998). Reactor models for horseradish peroxidase-catalyzed aromatic removal. *Journal of Environmental Engineering, ASCE* **124**(9): 794-802.

Caza, N., Bewtra, J. K., Biswas, N. and Taylor, K. E. (1999). Removal of phenolic compounds from synthetic wastewater using soybean peroxidase. *Water Research* **33**(13): 3012-3018.

Cooper, V. A. and Nicell, J. A. (1996). Removal of phenols from a foundry wastewater using horseradish peroxidase. *Water Research* **30**(4): 954-964.

Farhangrazi, Z. S., Copeland, B. R., Nakayama, T., Amachi, T., Yamazaki, I. and Powers, L. S. (1994). Oxidation-reduction properties of compound I and II of *Arthromyces ramosus* peroxidase. *Biochemistry* **33**(18): 5647-5652.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997). Optimization of the removal of phenol from wastewater catalyzed by *Arthromyces ramosus* peroxidase. *CSCE/ACSE Environ. Eng. Conf.*, Volume 2. Edmonton, Alberta, Canada: 1503-1510.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N. and Bewtra, J. K. (2001). Enzyme-catalyzed removal of phenol from refinery wastewater: Feasibility studies. *Water Environment Research* **73**(2): 165-172.

Ikehata, K. and Buchanan, I. D. (2002). Screening of *Coprinus* species for the production of extracellular peroxidase and evaluation of the enzyme for the treatment of aqueous phenol. *Environmental Technology* **23**(12): 1355-1367.

Kauffmann, C., Petersen, B. R. and Bjerrum, M. J. (1999). Enzymatic removal of phenols from aqueous solutions by *Coprinus cinereus* peroxidase and hydrogen peroxide.

Journal of Biotechnology **73**: 71-74.

Kinsley, C. and Nicell, J. A. (2000). Treatment of aqueous phenol with soybean peroxidase in the presence of polyethylene glycol. *Bioresource Technology* **13**(2): 139-146.

Kjalke, M., Andersen, M. B., Schneider, P., Christensen, B., Schulein, M. and Welinder, K. G. (1992). Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. *Biochimica et Biophysica Acta* **1120**: 248-256.

Klibanov, A. M., Alberti, B. N., Morris, E. D. and Felshin, L. M. (1980). Enzymatic removal of toxic phenols and anilines from waste waters. *Journal of Applied Biochemistry* **2**: 414-421.

Masuda, M., Sakurai, A. and Sakakibara, M. (2001a). Effect of reaction conditions on phenol removal by polymerization and precipitation using *Coprinus cinereus* peroxidase. *Enzyme and Microbial Technology* **28**: 295-300.

Masuda, M., Sakurai, A. and Sakakibara, M. (2001b). Effect of enzyme impurities on phenol removal by the method of polymerization and precipitation catalyzed by *Coprinus cinereus* peroxidase. *Applied Microbiology and Biotechnology* **57**: 494-499.

Nakamoto, S. and Machida, N. (1992). Phenol removal from aqueous solutions by peroxidase-catalyzed reaction using additives. *Water Research* **26**(1): 49-54.

Nicell, J. A., Bewtra, J. K., Biswas, N., Pierre, C. C. S. and Taylor, K. E. (1993). Enzyme catalyzed polymerization and precipitation of aromatic compounds from aqueous solution. *Canadian Journal of Civil Engineering* **20**: 725-735.

Sawai-Hatanaka, H., Ashikari, T., Tanaka, Y., Asada, Y., Nakayama, T., Minakata, H., Kunishima, N., Fukuyama, K., Yamada, H., Shibano, Y. and Amachi, T. (1995). Cloning, sequencing, and heterologous expression of a gene coding for *Arthromyces ramosus* peroxidase. *Bioscience, Biotechnology, and Biochemistry* **59**(7): 1221-1228.

Tanaka, M., Ishimori, K. and Morishima, I. (1999). Luminol activity of horseradish peroxidase mutants mimicking a proposed binding site for luminol in *Arthromyces ramosus* peroxidase. *Biochemistry* **38**(32): 10463-10473.

US EPA (1995). *Profile of the Petroleum Refining Industry.* EPA Office of Compliance Sector Notebook Project: EPA/310-R-95-013, U.S. Environmental Protection Agency, Washington, DC.

Villalobos, D. A. and Buchanan, I. D. (2002). Removal of aqueous phenol by *Arthromyces ramosus* peroxidase. *Journal of Environmental Engineering and Science* **1**: 65-73.

Wagner, M. and Nicell, J. A. (2001). Peroxidase-catalyzed removal of phenols from a petroleum refinery wastewater. *Water Science and Technology* **43**(2): 253-260.

Wagner, M. and Nicell, J. A. (2002). Impact of dissolved wastewater constituents on peroxidase-catalyzed treatment of phenol. *Journal of Chemical Technology and Biotechnology* **77**: 419-428.

Wright, H. and Nicell, J. A. (1999). Characterization of soybean peroxidase for the treatment of aqueous phenol. *Bioresource Technology* **70**(1): 69-79.

Wu, Y., Taylor, K. E., Biswas, N. and Bewtra, J. K. (1997). Comparison of additives in the removal of phenolic compounds by peroxidase-catalyzed polymerization. *Water Research* **31**(11): 2699-2704.

Zhang, G. and Nicell, J. A. (2000). Treatment of aqueous pentachlorophenol by horseradish peroxidase and hydrogen peroxide. *Water Research* **34**(5): 1629-1637.

Chapter 9. Summary and Conclusions

9.1. Screening of *Coprinus* species for extracellular peroxidase production

In this study, thirty-eight *Coprinus* species were evaluated to select the strains capable of producing large amounts of non-ligninolytic extracellular peroxidase that can be used for phenolic wastewater treatment. For the complete list of fungi, see Appendix 1. To examine their extracellular peroxidase production, liquid fermentation of *Coprinus* species was carried out in 250 mL shake flasks containing 100 mL of a medium composed of 1% glucose, 0.5% peptone, 0.3 % yeast extract and 0.3% malt extract (YM broth) at 25°C. The initial pH of liquid medium was 6.2.

Peroxidase activity was detected in the cultures of five strains of *C. cinereus* UAMH 4103, UAMH 7907, IFO 8371, IFO 30114 and IFO 30116, *C. lagopus* UAMH 7499, *C. echinosporus* NBRC 30630 (IFO 30630), *C. macrocephalus* NBRC 30117 (IFO 30117), and unidentified *Coprinus* sp. UAMH 358, UAMH 380, UAMH 10065, UAMH 10066, UAMH 10067 and 074, as well as of *Arthromyces ramosus* FERM BP-838. Of these fungi, three strains of *C. cinereus* UAMH 4103, UAMH 7907 and IFO 30116 and one *Coprinus* sp. UAMH 10067 were studied further for peroxidase production.

Peroxidase activity was detected after 3 days of growth and had reached its peak another 5 to 6 days later. The peroxidase activity appeared to increase with a corresponding depletion of glucose concentration. Although the peroxidase activity rapidly declined after the exhaustion of glucose in cultures of three *C. cinereus* strains, it was stable for several days in that of *Coprinus* sp. UAMH 10067.

The highest average peroxidase activity was found in the cultures of *C. cinereus*

UAMH 4103 (28.3 U mL^{-1}); however, there was no significant difference at the 95% confidence interval in the maximum peroxidase activity among the cultures of three *C. cinereus* (UAMH 4103, UAMH 7907 and IFO 30116). Although the maximum peroxidase activity in the cultures of *Coprinus* sp. UAMH 10067 (15 U mL^{-1}) was lower than those of *C. cinereus* strains, the peroxidase produced by the former fungus appeared to be more stable than those produced by the latter ones. Thus, the peroxidases produced by one of the *C. cinereus* strains, UAMH 4103, and *Coprinus* sp. UAMH 10067 were chosen for further comparative studies for their applications to phenolic wastewater treatment, as well as for the peroxidase production optimisation studies.

In this study, a large number (38 strains) and a wide variety (17 known species + 14 unknown species) of *Coprinus* species were evaluated. However, only a limited number of *Coprinus* species (4 known species + 6 unknown species) were found to produce extracellular peroxidase. Peroxidase production by *C. cinereus* was previously reported (Morita *et al.*, 1988) and also observed in this study. Peroxidase production by three other known *Coprinus* species, including *C. lagopus* UAMH 7499, *C. echinosporus* NBRC 30630 (IFO 30630) and *C. macrocephalus* NBRC 30117 (IFO 30117), was demonstrated for the first time, although the enzyme activity was relatively minor as compared with that of *C. cinereus*. As discussed in Chapter 5, neither taxonomic classification nor fungal ecology of *Coprinus* species can provide a clue to explain this observation. In addition, the physiological roles of extracellular peroxidase produced by some of the *Coprinus* species are also unknown. It can be important to know the association between peroxidase production and fungal ecology, physiology or development in their natural habitat, because there may be some rational ways to improve *Coprinus* peroxidase production, such as using their metabolites as chemical inducers and using natural substrates to stimulate the enzyme production. Similar approaches have been used for LiP, MnP and laccase productions as reviewed in Chapter 3.

As a practical way to screen potential *Coprinus* species for extracellular peroxidase production from the field, urea treatment of soil, which had been developed for mycological research, was proven to be a good method in this study. There may be *Coprinus* species that are capable of producing environmentally and industrially important extracellular peroxidases more efficiently than the known strains, or that are capable of producing enzymes with unique properties, or growing and producing enzymes under unique environment, such as in cold or hot climates that may suit their production and application in a particular region of the world. Thus, more screening studies can be done based on the findings of this study.

9.2. Purification and characterisation of *Coprinus* peroxidase

Two non-ligninolytic extracellular fungal peroxidases obtained by liquid fermentation of *C. cinereus* UAMH 4103 and *Coprinus* sp. UAMH 10067 were purified and characterised. The crude peroxidases in culture filtrates were concentrated by ultrafiltration and purified with anion-exchange chromatography, size-exclusion chromatography and FPLC. The RZ value, a purity index for haemoproteins ($A_{405\ nm}/A_{280\ nm}$), of purified peroxidases were 2.5 and 2.3 for the enzymes from *C. cinereus* UAMH 4103 and *Coprinus* sp. UAMH 10067, respectively.

The purified *Coprinus* peroxidases have a molecular weight of around 36 kDa based on MALDI-TOF mass spectrometry. The purified *Coprinus* peroxidases contained multiple isoenzymes with slight variations in molecular weights, which suggests that the degrees of glycosylation were different among the isoenzymes. The amino acid composition analyses of two purified *Coprinus* peroxidases revealed that these two enzymes had very similar amino acid compositions, and that they were also similar to the previously studied CIP from different strains and ARP.

Catalytic properties of two *Coprinus* peroxidases were nearly identical: both enzymes were active in the pH range of 5 to 10 and showed an optimum pH at pH 6 using phenol as a substrate; and the shapes of hydrogen peroxide concentration versus peroxidase activity plots were identical for both enzymes. In addition, purification of *Coprinus* peroxidase had little impact on the catalytic activities of these enzymes. This fact encourages the use of crude peroxidase for wastewater treatment because enzyme purification substantially increases the production cost.

Some kinetic parameters, which are important for phenolic wastewater treatment, were determined by applying a model developed for plant peroxidases. Comparison of the estimated kinetic parameters for *Coprinus* peroxidases with published data indicates that the *Coprinus* peroxidases are catalytically faster than HRP and SBP in hydrogen peroxide uptake and phenol oxidation, although the former enzymes are more susceptible to the inhibition by excess hydrogen peroxide than are the latter plant peroxidases.

Although the catalytic properties of two *Coprinus* peroxidases were very similar, thermal and pH stabilities of these enzymes were substantially different. The peroxidase from *Coprinus* sp. UAMH 10067 was more stable than that from *C. cinereus* UAMH 4103 at an elevated temperature (50°C) and under weakly acidic (pH 4) and weakly basic (pH 10) conditions. Higher thermal and pH stabilities of the peroxidase from *Coprinus* sp. UAMH 10067 suggest the suitability of this enzyme for industrial wastewater treatment, in which the temperature and pH of the wastewater tend to fluctuate during the operation.

Enzyme purification also showed some impact on the stability of *Coprinus* peroxidases: in general, the crude enzymes were more stable at elevated temperature, but were less stable at 25°C under acidic and basic conditions than the purified ones. Filtrates of *Coprinus* liquid cultures were used as sources of crude enzymes. Higher thermal stability of crude *Coprinus* peroxidase can be advantageous for its application to wastewater treatment. Although the lesser pH stability of crude enzymes may be unfavourable, the long-term (a 5-day period) pH stability investigated in this study is unlikely important for actual phenol treatment because the fungal peroxidase-catalysed phenol removal is a relatively fast process (several minutes to a few hours).

9.3. Evaluation of *Coprinus* peroxidase for aqueous phenol treatment

The crude and purified peroxidases produced by *C. cinereus* UAMH 4103 and *Coprinus* sp. UAMH 10067 were evaluated for batch treatment of aqueous phenol in various pH buffers at 25°C. Both enzymes performed equally well in aqueous phenol treatment around neutral pH (pH 6 to 8); however, the peroxidase from *Coprinus* sp. UAMH 10067 worked better at pH 9 than did that from *C. cinereus* UAMH 4103. The broader working pH of the former enzyme can be another advantage for its application to industrial wastewater treatment.

The effect of H_2O_2 concentration on the aqueous phenol treatment was studied using crude peroxidase from *C. cinereus* UAMH 4103. The stoichiometry between H_2O_2 consumption and phenol removal during the aqueous phenol treatment was close to one, which is consistent with the previously reported values for other peroxidases. It was also observed that the addition of PEG or chitosan did not affect the stoichiometry.

Crude *Coprinus* peroxidase containing soluble residues derived from liquid fungal cultures performed much better than purified *Coprinus* peroxidase. When crude enzymes were used, the enzyme requirements to achieve 95% removal of initial phenol (1.1 mM) were 15 to 18 times smaller than those when purified ones were used (at pH 7). It was suggested that biopolymers such as polysaccharides and proteins in the crude peroxidase solution might protect the enzyme molecules from entrapments by polymeric products arising from phenol oxidation and subsequent radical coupling.

As previously reported for other plant and fungal peroxidases, addition of PEG, as well as of chitosan, also enhanced the phenol removal catalysed by *Coprinus* peroxidase. It was found that PEG was more effective than chitosan in the enhancement of *Coprinus* peroxidase-catalysed treatment of aqueous phenol. Although PEG addition reduced the enzyme requirement of either purified or crude *Coprinus* peroxidase, there was no difference between the enzyme requirements in the presence of PEG in both cases (at pH 7). This fact implies that the effects of soluble fungal residues and PEG are very similar, and they do not act synergistically. Because both fungal residues and PEG would contribute additional oxygen demands to wastewater, their addition needs to be minimised. Partial purification of peroxidase, using ultrafiltration to remove smaller organic and inorganic molecules from crude peroxidase solution, and/or controlled addition of PEG may be recommended depending on the operational requirements.

Comparison of the phenol removal efficiencies of *Coprinus* peroxidase to those of previously studied, commercially available plant and fungal peroxidases, including HRP, SBP and ARP, revealed that although they were comparable in the presence of PEG, crude *Coprinus* peroxidase was superior in phenol removal to those enzymes in the absence of PEG. The use of crude enzyme is generally preferable, because it will reduce the enzyme production cost substantially, although partial purification of enzyme may be needed as discussed above. Thus, it can be suggested that the crude *Coprinus* peroxidases, particularly that from *Coprinus* sp. UAMH 10067, have a great potential for their applications to phenolic wastewater treatment.

9.4. Optimisation of *Coprinus* peroxidase production

In order to maximise the extracellular peroxidase production by *Coprinus* species, the culture conditions were optimised through a series of batch growth experiments in 500 mL shake flasks. Based on the results of studies summarised above, two *Coprinus* species, *C. cinereus* UAMH 4103 and *Coprinus* sp. UAMH 10067 were selected for peroxidase production. The optimisation of growth conditions for *Coprinus* peroxidase production consisted of four steps, including screening of carbon and nitrogen sources, screening of important factors, optimisation of selected important factors, and confirmation of optimised growth conditions. The YM broth used in fungal screening studies (Chapters 4 and 5) was used as a basal medium and was modified through the optimisation experiments.

Varieties of carbon and nitrogen sources were tested to determine the best compounds to support peroxidase production by *Coprinus* sp. UAMH 10067. Of the carbon sources examined, glucose and fructose were found to support well the growth and peroxidase production of this fungus. Peptone and casitone were found to be the best nitrogen sources. Based on the economical consideration, glucose and peptone were subsequently selected for carbon and nitrogen sources, respectively.

Seven potentially important factors for *Coprinus* peroxidase production were screened using a 2^{7-3}_{IV} fractional factorial design experiment. The factors evaluated include: 1) glucose concentration, 2) nitrogen source concentration, 3) yeast extract concentration, 4) malt extract concentration, 5) type of nitrogen source (peptone or casitone), 6) addition of 2 mg L^{-1} $FeSO_4$, and 7) initial medium pH. Of these potential factors, the main effects of two factors, including glucose and nitrogen source concentrations, were found to be significant. Thus, these factors were selected for further optimisation.

The concentrations of glucose and peptone were optimised for peroxidase production by two *Coprinus* species using 2-factor central composite design experiments. The optimised glucose and peptone concentrations were determined by regression analyses as 2.7 % and 0.8% for *Coprinus* sp. UAMH 10067, and 2.9% and 1.4% for *C. cinereus* UAMH 4103, respectively. Using the optimised culture media, the maximum peroxidase activities achieved in the confirmation growth experiments were 34.5 ± 1.5 U mL^{-1} for *Coprinus* sp. UAMH 10067 and 68.0 ± 3.1 U mL^{-1} for *C. cinereus* UAMH 4103, which are more than a 2-fold increase as compared to the peroxidase activities achieved under the initial growth conditions.

9.5. Treatment of oil refinery wastewater using crude *Coprinus* peroxidase

Crude extracellular peroxidase from *C. cinereus* UAMH 4103 was evaluated for its application to the treatment of a strong oil refinery wastewater containing phenolic compounds in high concentrations. The refinery wastewater was collected from a drier unit of the catalytic treatment process, in which phenolic compounds generated in catalytic cracking operation was removed from petroleum distillates by the addition of calcium chloride and sodium chloride along with moisture and other impurities.

Culture filtrates of *C. cinereus* UAMH 4103 was used as a source of crude *C. cinereus* peroxidase (CIP). Diluted refinery wastewater containing 6.4 mM of total phenol was treated with the crude CIP as well as with purified commercial enzymes, including ARP and HRP, in order to compare the phenol removal efficiencies. Commercial ARP was used to examine the effect of enzyme purification, because this fungal enzyme has identical structural and catalytic properties to those of previously studied CIP (Kjalke *et al.*, 1992).

The effects of pH and H_2O_2 concentration on the phenol removal from the refinery wastewater were studied using crude CIP. The optimum pH for phenol removal was observed around pH 7, which is consistent with the results obtained in aqueous phenol treatment (Chapter 5). More H_2O_2 was consumed during the refinery wastewater treatment than that required for aqueous phenol treatment, which is considered to be due to the oxidation of reduced sulphur compounds in the refinery wastewater that exerts additional H_2O_2 demand.

Unlike the treatment of buffered aqueous phenol, the precipitation of polymer products was not observed, but the formation of colloidal coloured products was observed in the oil refinery wastewater treated with peroxidase and H_2O_2. The coloured products could be removed by alum coagulation. Neither addition of PEG nor purity of fungal peroxidase (crude CIP and purified ARP) had effect on the phenol removal, which is also different from the observations made in aqueous phenol treatment. However, phenol removal was improved by the PEG addition when purified HRP was used as a peroxidase. The observed phenol removal efficiencies were in the following order: purified HRP with PEG > crude CIP or purified ARP (with or without PEG) > purified HRP without PEG.

The use of crude CIP is likely a viable option for the refinery wastewater treatment because of its potentially low production cost and lack of the requirement of PEG addition or enzyme purification.

Although the omission of enzyme purification may be beneficial for the cost reduction, the crude CIP contained significant amounts of organic impurities that would contribute additional oxygen demands to the wastewater and cause some impacts on the downstream treatment process or the environment. Thus, the fate of these impurities during the refinery wastewater treatment was studied using BOD_5 and COD as measures of the oxygen demands. Although both BOD_5 and COD were reduced by 52% and 58%, respectively, after the enzymatic treatment ($[CIP]_0 = 2$ U mL^{-1}) and subsequent alum coagulation of refinery wastewater containing 6.4 mM total phenol, the organic impurities in crude CIP were not apparently removed either by enzymatic treatment or by alum coagulation. This result suggests that, again, partial purification using ultrafiltration is recommended for the actual application of crude *Coprinus* peroxidase to wastewater treatment.

9.6. Conclusions

A series of investigations conducted for this thesis successfully demonstrated the viability of non-ligninolytic extracellular peroxidase production by selected *Coprinus* species and subsequent application of crude peroxidase to phenolic wastewater treatment. The following specific conclusions were drawn from the results of this study:

(1) Several potential *Coprinus* species, including *C. cinereus* UAMH 4103,

UAMH 7909 and IFO 30116 and *Coprinus* sp. UAMH 10067, were found to produce pronounced amounts of non-ligninolytic extracellular peroxidases that were applicable to phenolic wastewater treatment.

(2) The extracellular peroxidases produced by two of the potential fungi, *C. cinereus* UAMH 4103 and *Coprinus* sp. UAMH 10067, were purified and characterised. The molecular weight of these peroxidases was determined as 36 kDa based on MALDI-TOF mass spectrometry. The amino acid compositions of these enzymes were found to be very similar to each other. The compositions are also similar to those of previously studied non-ligninolytic fungal peroxidases including CIP from different strains and ARP.

(3) Whereas the catalytic properties of two *Coprinus* peroxidases were found to be nearly identical, the stabilities of these enzymes were substantially different. The peroxidase from *Coprinus* sp. UAMH 10067 was more stable at high temperature (50°C) and under weakly acidic (pH 4) and basic (pH 10) conditions than that from *C. cinereus* UAMH 4103. The enzyme purification had little effects on the catalytic properties of two *Coprinus* peroxidases; however, it enhanced pH stability, but lowered thermal stability of these enzymes.

(4) The extracellular peroxidase from *Coprinus* sp. UAMH 10067 was found to have a broader working pH for aqueous phenol treatment than that from *C. cinereus* UAMH 4103, although both enzymes performed equally well around neutral pH. The phenol removal efficiencies of *Coprinus* peroxidases were comparable to those of

previously studied plant and fungal peroxidases in the presence of PEG.

(5) It was demonstrated that use of crude *Coprinus* peroxidases for aqueous phenol treatment reduced the enzyme requirements (>95% removal of 1.1 mM phenol) by the factor of 15 to 18 as compared to when purified *Coprinus* peroxidases were used. The addition of PEG enhanced the phenol removal further, which implied that the effect of impurities in crude *Coprinus* peroxidases on the phenol removal was similar to that of PEG.

(6) Batch productions of extracellular peroxidase by *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103 were successfully optimised through a series of growth experiments. With optimised growth media, maximum *Coprinus* peroxidase activities were increased more than double as compared to those with the original medium for both *Coprinus* species, 34.5 ± 1.5 U mL^{-1} for *Coprinus* sp. UAMH 10067 and 68.0 ± 3.1 U mL^{-1} for *C. cinereus* UAMH 4103.

(7) The applicability of crude *Coprinus* peroxidase to the treatment of strong oil refinery wastewater containing high concentrations of phenolic compounds was successfully demonstrated. Total phenol and oxygen demand were reduced by enzymatic treatment and subsequent alum coagulation. Unlike the cases of aqueous phenol treatment, neither the impurities in crude enzyme nor PEG addition had an effect on the phenol removal from the refinery wastewater.

(8) It was found that the impurities in crude *Coprinus* peroxidase apparently

remained in the enzymatically treated oil refinery wastewater even after coagulation. This suggests that the partial purification of *Coprinus* peroxidase may be recommended to reduce the impact of additional organic contents derived from the crude enzyme on the downstream treatment process or environment.

9.7. Recommendations

Based on the findings from this study, the following research areas have been identified for further investigation:

(1) Because the number of *Coprinus* species evaluated in this study is still limited, there may be possibilities of finding more potential fungi for the production of non-ligninolytic extracellular peroxidase. Evaluation of *Coprinus* species collected from different parts of the world may lead the discovery of unique peroxidase produced by such fungi. Isolation and evaluation of the fungi from the sites previously contaminated by substances containing phenols (such as oil and bitumen) may also be advised. Utilisation of different screening methods, such as qualitative *in vitro* activity assay, immunoblotting (Orth *et al.*, 1993) and peroxidase cDNA probe (Kimura *et al.*, 1990), can be recommended for increased screening efficiency in the detection of peroxidase production.

(2) Evaluation of inducers to stimulate the production of *Coprinus* peroxidase, which is an approach similar to that often used for fungal laccase production, may be recommended. In order to reduce the enzyme production cost, studying the use of waste

products and natural solid substances as growth substrates may also be encouraged. It may also be important to study fungal physiology of *Coprinus* species producing non-ligninolytic peroxidase in order to find rational approaches to select appropriate inducer chemicals or growth substrates.

(3) The scale-up of *Coprinus* peroxidase production in larger bioreactors is recommended for study. A fed-batch process to avoid the inhibition effects of high glucose and peptone concentrations is recommended. Evaluation of novel bioreactor configurations, such as membrane bioreactors (Govender *et al.*, 2003), for continuous production of *Coprinus* peroxidase may also be advised.

(4) Optimised purification of crude *Coprinus* peroxidase to minimise the introduction of additional organic loads to wastewater is recommended to study. Because bio-macromolecules such as polysaccharides and proteins in crude peroxidase preparations are believed to be protective to the enzyme during the phenolic wastewater treatment, the removal of smaller organic molecules from crude enzyme by ultrafiltration may be effective for such a purpose. Analyses of molecular weight ranges of these bio-macromolecules may be helpful for optimising enzyme purification.

(5) Treatment of various real wastewaters containing diverse phenolic compounds, such as pulp mill effluents, using crude *Coprinus* peroxidase may be needed to demonstrate the viability of *Coprinus* peroxidase further. The effects and fates of other constituents in real wastewaters during the *Coprinus* peroxidase-catalysed treatment may be important to study.

(6) Characterisation of and risk assessment on the reaction products, including both water-soluble and water-insoluble products, of *Coprinus* peroxidase-catalysed treatment of phenolic wastewater will be definitely needed. For residual water-soluble products, toxicity assessments using the Microtox® acute toxicity assay (Aitken *et al.*, 1994; Ikehata and Nicell, 2000) is recommended. In addition to the acute toxicities, the assessments of chronic toxicities such as carcinogenicity and mutagenicity, as well as developmental and reproductive toxicities may also be needed for the peroxidase-treated wastewaters. Some tests on the biodegradability and the possibility of bioaccumulation of the soluble products may be required as well. Stability tests and subsequent disposal must also be studied for solid precipitates.

References

Aitken, M. D., Massey, I. J., Chen, T. and Heck, P. E. (1994). Characterization of reaction products from the enzyme catalyzed oxidation of phenolic pollutants. *Water Research* **28**(9): 1879-1889.

Govender, S., Jacobs, E. P., Leukes, W. D. and Pillay, V. L. (2003). A scalable membrane gradostat reactor for enzyme production using *Phanerochaete chrysosporium*. *Biotechnology Letters* **25**: 127-131.

Ikehata, K. and Nicell, J. A. (2000). Color and toxicity removal following tyrosinase-catalyzed oxidation of phenols. *Biotechnology Progress* **16**(4): 533-540.

Kimura, Y., Asada, Y. and Kuwahara, M. (1990). Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. *Applied Microbiology and Biotechnology* **32**: 436-442.

Kjalke, M., Andersen, M. B., Schneider, P., Christensen, B., Schulein, M. and Welinder, K. G. (1992). Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. *Biochimica et Biophysica Acta* **1120**: 248-256.

Morita, Y., Yamashita, H., Mikami, B., Iwamoto, H., Aibara, S., Terada, M. and Minami, J. (1988). Purification, crystallization, and characterization of peroxidase from *Coprinus cinereus*. *Journal of Biochemistry* **103**(4): 693-699.

Orth, A. B., Royse, D. J. and Tien, M. (1993). Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. *Applied and Environmental Microbiology* **59**(12): 4017-4023.

Appendices

Appendix 1. Description of Fungi

Table A1.1. Description of fungi.

Name	Accession number	Description
Arthromyces ramosus N. Amano	FERM BP-838	Obtained from National Institute of Bioscience and Human Technology, Ibaraki, Japan.
Coprinus atramentarius (Bull.:Fries) Fries	UAMH 7496	Isolation Data: ex basidiospores from fruiting body at base of Populus tremuloides, Spruce Grove, Alta., S.P. Abbott (SA 900) 23 Aug., 1993.
Coprinus cinereus (Schaeffer : Fries) S.F. Gray	NBRC 8371[*] (IFO 8371)	f. *microsporus* (Hongo) (1965) (*Coprinus macrorhizus* f. *microsporus*) IFO (K. Tubaki).
	NBRC 30114[*] (IFO 30114)	(1976) Dept. Bot., Univ. Edinburgh (R.F.O. Kemp; H1/ dikaryon; cucumber houses).
	NBRC 30116[*] (IFO 30116)	(1976) Dept. Bot., Univ. Edinburgh (R.F.O. Kemp; PO1 X O2 dikaryon) G. Butler, germinating beet seed.
	UAMH 4103	Isolation Data: beet seed Birmingham Butler, Apr. 1973, Sender: Watling, R. P/04 NAS mono.
	UAMH 7907	Isolation Data: ex air, municipal landfill site Sosnowiec, Poland K. Ulfig, 11 Oct. 1993 (morph *Hormographiella aspergillata*), Sender: Ulfig, K. IEIA 511.
Coprinus clastophyllus Maniotis	UAMH 4104	Isolation Data: original type isolate ex Maniotis USA Maniotis, Sender: Watling, R. C/03 mono.

Note: [*] These fungi are currently distributed by NITE Biological Resources Center (NBRC), National Institute of Technology and Evaluation, Chiba, Japan.

Table A1.1. Description of fungi (continued).

Name	Accession number	Description
Coprinus comatus (Mull.:Fries) S.F.Gray	UAMH 7498	Isolation Data: basidiospores from fruiting body on lawn nr Populus sp., Devonian Botanic Garden, Devon, Alta. S.P. Abbott (SA 934) 10 Sep., 1993.
Coprinus domesticus Fries	UAMH 4101	Isolation Data: poss ex CBS Holland Ginns, Sender: Watling, R. H/1
Coprinus echinosporus Buller	NBRC 30630* (IFO 30631)	(1979) Biol. Lab., Yoshida College, Kyoto Univ. (N. Sagara, NS 581). Source: urea-treated ground in *Pinus densiflora-Chamaecyparis obtuse* forest.
	NBRC 30631* (IFO 30631)	(1979) Biol. Lab., Yoshida College, Kyoto Univ. (N. Sagara, NS 582). Source: urea-treated ground in *Castanopsis cuspidate* forest.
Coprinus kimurae Hongo & Aoki	UAMH 4105	Isolation Data: Tokorozawa City, Japan, Aoki, 28 May 1978, Sender: Watling, R. 504/05 mono.
Coprinus lagopides Karsten	NBRC 30120* (IFO 30117)	(1976) Dept. Bot., Univ. Edinburgh (R.F.O. Kemp, K/1 dikaryon) <= J. Daams. Source: Pure sand.
Coprinus lagopus (Fr.) Fr.	UAMH 7499	Isolation Data: ex basidiospores from fruiting body on woodchip trail Devonian Botanic Garden, Devon, Alta.; S.P. Abbott (SA 893) 16 Aug., 1993.
Coprinus macrocephalus nom. prov.	NBRC 30117* (IFO 30117)	(1976) Dept. Bot., Univ. Edinburgh (R.F.O. Kemp, E/1 dikaryon) <= P.D. Orton. Source: old bull or cow dung.

Note: * These fungi are currently distributed by NITE Biological Resources Center (NBRC), National Institute of Technology and Evaluation, Chiba, Japan.

Table A1.1. Description of fungi (continued).

Name	Accession number	Description
Coprinus micaceus (Bull.:Fr.) Fr.	UAMH 7500	Isolation Data: ex basidiospores from fruiting body at base of Populus tremuloides (aspen poplar) Spruce Grove, Alta. S.P. Abbott (SA 902) 23 Aug., 1993.
Coprinus neolagopus Hongo & Sagara	NBRC 30476* (IFO 30476)	(1978) Biol. Lab., Yoshida College, Kyoto Univ. (N. Sagara, NS 556). Source: soil treated with urea. Ammonia fungus.
	NBRC 32947* (IFO 32947)	(1997) Grad. Sch. Human & Environm. Stud., Kyoto Univ. (N. Sagara, NAO 653). Source: forest ground dressed with oxamide.
Coprinus phlyctidosporus Romagnesi	NBRC 30478* (IFO 30478)	(1978) Biol. Lab., Yoshida College, Kyoto Univ. (N. Sagara, NS 559). Source: soil treated with urea. Ammonia fungus.
	NBRC 32946* (IFO 32946)	(1997) Grad. Sch. Human & Environm. Stud., Kyoto Univ. (N. Sagara, NAO 665). Source: urea-treated soil in forest.
Coprinus psychromorbidus Redhead & Traquair	UAMH 6383	Isolation Data: alfalfa 1979, Sender: Ayer, W.A. LRS-067, Strain Characters: psychrophilic low temperature basidiomycete. Does not grow at room temperature. Optimum 18°C.
Coprinus radiatus (Bolton:Fries) Fries	NBRC 30118* (IFO 30118)	(1976) Dept. Bot., Univ. Edinburgh (R.F.O. Kemp, AO4 X O5 dikaryon).
Coprinus sterquilinus Fries	UAMH 4155	Isolation Data: Braunton Burrows, Soms H. Simpson, 20 Jun., 1972, Sender: Kemp, R. B/01.

Note: * These fungi are currently distributed by NITE Biological Resources Center (NBRC), National Institute of Technology and Evaluation, Chiba, Japan.

Table A1.1. Description of fungi (continued).

Name	Accession number	Description
Coprinus velox Godey	UAMH 4137	Isolation Data: Braunton Burrows Webster 27 Sep 1974, Sender: Walting, R. KCC 243/03 mono.
Coprinus sp.	UAMH 358	Isolation Data: ex stool Edmonton, Alta. Carmichael, J.W. 1955.
	UAMH 380	Isolation Data: specimen Edmonton, Alta. Carmichael, J.W. 1955.
	UAMH 1211	Isolation Data: soft rot cedar cooling tower Toronto Carmichael, J.W. 1962.
	UAMH 3029	Isolation Data: sputum UCLA Medical Centre, Sender: Pore, R.S., Cross Ref. Nos.: Orr O-2563.
	UAMH 3261	Isolation Data: dung ex rodent Baja, Calif. R. Benjamin 22 Feb 1963, Sender: Benjamin, R.K. 1777.
	UAMH 4568	Isolation Data: soil, Sender: Guarro, J. FFBA 222.
	UAMH 6478	Isolation Data: adult Dendroctonus ponderosae beetle Valemount, B.C. Yamoaka, Y. 8 Aug 1988, Sender: Yamaoka, Y. NOF 1395, Strain Characters: pigment tan.
	UAMH 7251	Isolation Data: ex bronchial washing, female 80 yr Alberta 1992, Sender: Rennie, R. MY 3333, Strain Characters: cycloheximide sensitive. // Benomyl resistant.
	UAMH 7412	Isolation Data: outdoor air ex RCS strip Edmonton, Alta. S.P. Abbott (EA-1-1J) 14 Jul 1993, Sender: Dhanani, N. 1, Strain Characters: pigment brown.

Table A1.1. Description of fungi (continued).

Name	Accession number	Description
Coprinus sp.	UAMH 7413	Isolation Data: indoor air ex RCS strip Edmonton, Alta. S.P. Abbott (EA-2-2G) 14 Jul 1993, Sender: Dhanani, N. 2, Strain Characters: pigment brown, occasional blue-green patches.
	UAMH 10065	Isolation Data: urea baited soil, Alberta A. Suzuki 2001, Sender: Suzuki, A. 074UA.
	UAMH 10066	Isolation Data: urea baited soil, Alberta A. Suzuki 2001, Sender: Suzuki, A. 018UA.
	UAMH 10067	Isolation Data: urea baited soil, Alberta A. Suzuki 2001, Sender: Suzuki, A. 043UA.
	074	From urea treated soil, Alberta, Suzuki, A. 2001. (= UAMH 10065).

Contact Information (As of August 1st, 2003)

University of Alberta Microfungus Collection and Herbarium (UAMH)

Lynne Sigler, Curator & Professor

Arlene L. Flis, Assistant Curator

Teleph

FAX: (

Postal address: University of Alberta Microfungus Collection & Herbarium (UAMH),

NITE Biological Resource Center (NBRC)

(Current distributor of IFO microorganisms)

Department of Biotechnology

National Institute of Technology and Evaluation

International Patent Organism Depositary

(*Arthromyces ramosus* FERM BP-838)

National Institute of Advanced Industrial Science and Technology

Appendix 2. Production of Ligninolytic Enzymes by *Coprinus* Species

In this study, the activities of ligninolytic (lignin-degrading) enzymes including laccase, LiP and MnP in the liquid cultures of *Coprinus* species were also monitored. Although MnP was not detected in the any cultures of *Coprinus* species evaluated in this study, it was found that some *Coprinus* species produced either laccase or LiP. As shown in Figures A2.1 and A2.2, the highest average laccase activity of 0.1 U mL^{-1} was found in the culture broth of *C. radiatus* IFO 30118 on day 10. Moderate laccase activity was also found in the cultures of *C. macrocephalus* IFO 30117, *C. phlyctidosporus* IFO 30478, *C. lagopus* UAMH 7499, *C. atramentarius* UAMH 7496, *C. sterquilinus* UAMH 4155, and *Coprinus* spp. UAMH 6478, UAMH 10065, UAMH 10066 and 074.

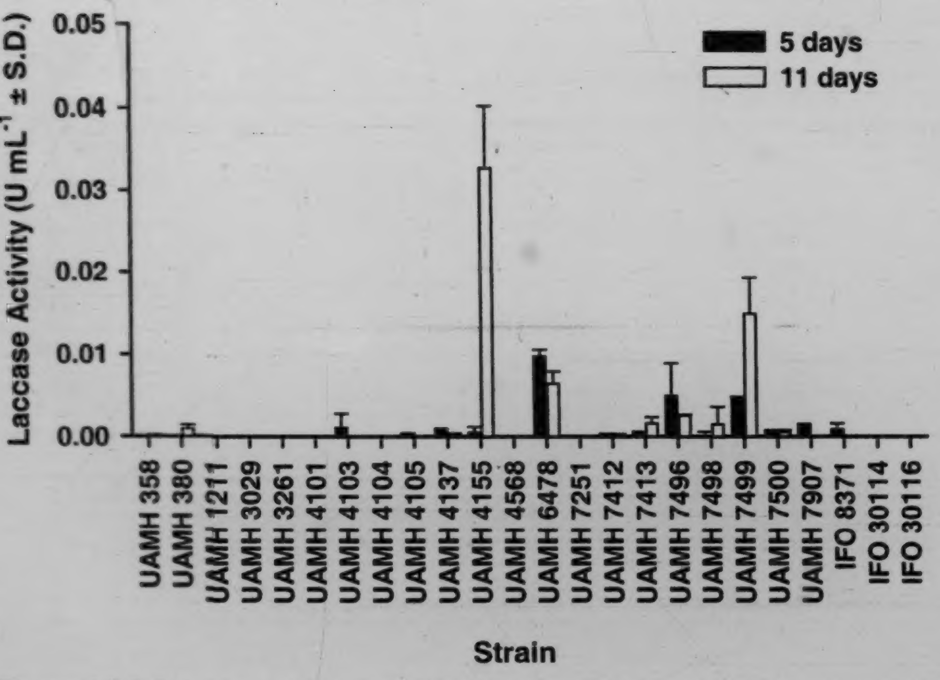


Figure A2.1. Laccase activity in the liquid culture (YM broth) of *Coprinus* species grown at 25°C.

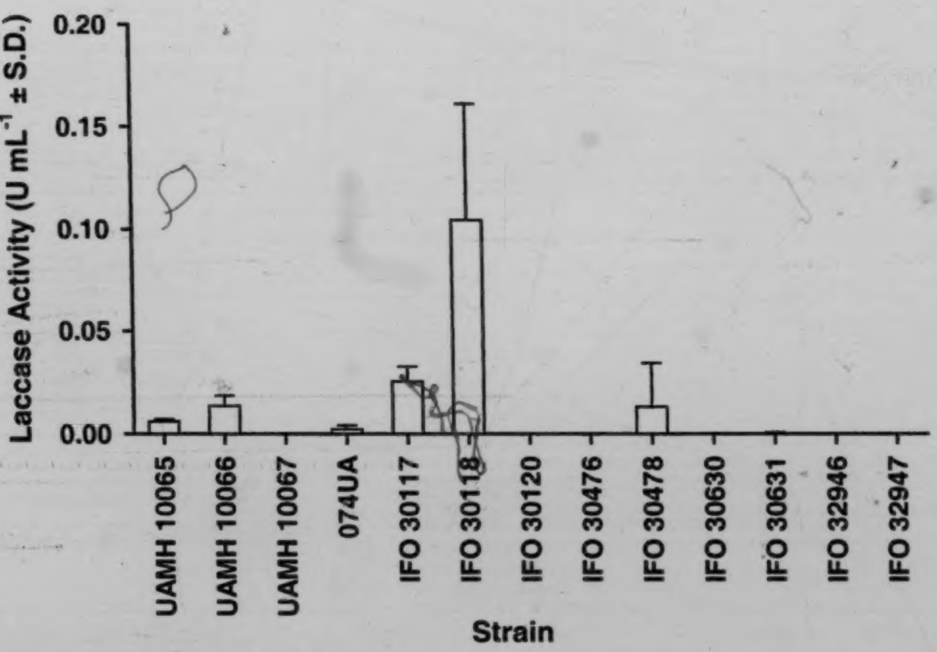


Figure A2.2. Laccase activity in the liquid culture (YM broth) of *Coprinus* species grown at 25°C for 10 days.

Figure A2.3 shows the production of LiP by *Coprinus* species in YM broth. The highest LiP activity of 0.026 ± 0.014 U mL^{-1} was found in the culture of *C. velox* UAMH 4137 after 11 days. Weak LiP activity was also found in the cultures of *Coprinus* spp. UAMH 7251 and UAMH 7418.

Although these ligninolytic enzymes are also useful for the environmental applications (see Chapters 2 and 3), it is considered that the activities of these enzyme found here are too low. Thus, no further study was conducted on the production of these enzymes by *Coprinus* species.

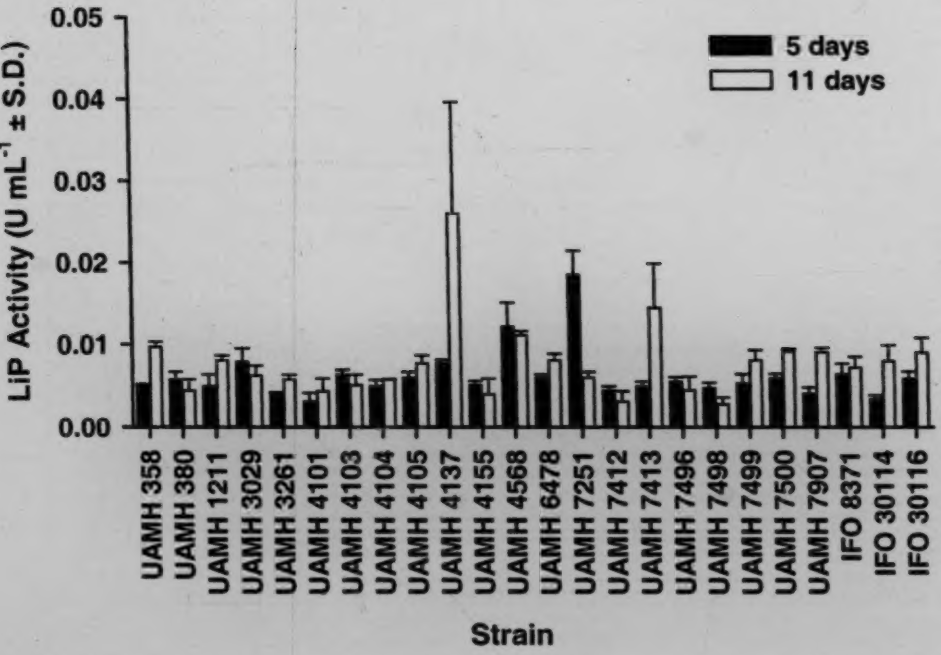


Figure A2.3. LiP activity in the liquid culture (YM broth) of *Coprinus* species grown at 25°C.

Appendix 3. Tabulated Data for Regression Analyses

A3.1. Psudo-steady state model for peroxidase activity (Chapter 6, Figure 6.4)

Assuming steady-state hydrogen peroxide consumption during the peroxidase activity assay, the relationship between initial hydrogen peroxide concentration and the apparent peroxidase activity (*i.e.*, hydrogen peroxide consumption rate) can be modelled using the following equation developed by Nicell and Wright (1997):

$$\frac{d[\mathrm{H_2O_2}]}{dt} = \frac{-E_0}{\left(a + \frac{b}{[\mathrm{H_2O_2}]} + c[\mathrm{H_2O_2}]\right)} \qquad \text{(A3-1)}$$

where E_0 is total peroxidase concentration. The constants *a*, *b* and *c* are defined by

$$a = \frac{1}{2[\mathrm{Phenol}]_s}\left(\frac{1}{k_2} + \frac{1}{k_3}\right)$$

$$b = \frac{1}{2k_1}$$

$$c = \frac{k_{app}}{2k_3[\mathrm{Phenol}]_s(k_a + k_b[\mathrm{Phenol}]_s)}$$

where k_1, k_2, k_3, k_a, k_b and k_{app} are kinetic rate constants shown in Figure 6.1, and $[\mathrm{Phenol}]_s$ is the assay phenol concentration ($= 1 \times 10^{-2}$ M). It should be noted that inactivation of peroxidase through P-670 formation and consumption of hydrogen peroxide through the upper catalytic cycle (– Compound I – Compound I-H_2O_2 – Resting Enzyme –) in Figure 6.1, are insignificant in the presence of phenolic substrate (Nicell, 1994); thus they are neglected in the model. The relative activity (*R*) of peroxidase is independent of E_0 and can be expressed as:

$$R = \left(\alpha + \frac{\beta}{[H_2O_2]} + \gamma [H_2O_2] \right)^{-1} \qquad \text{(A3-2)}$$

where the parameters α, β and γ are related to the constants *a*, *b* and *c* as follows:

$$\frac{a}{\alpha} = \frac{b}{\beta} = \frac{c}{\gamma} \qquad \text{(A3-3)}$$

$$a = \frac{KC_a}{\left(1 + \frac{\beta}{\alpha [H_2O_2]_s} + \frac{\gamma}{\alpha} [H_2O_2]_s \right)} \qquad \text{(A3-4)}$$

where *K* is the unit conversion factor (= 6×10^7 U s mol^{-1}), C_a is the proportionality constant relating the molar concentration of peroxidase to the corresponding activity, and $[H_2O_2]_s$ is the standard assay hydrogen peroxide concentration (= 2×10^{-4} M). The C_a value for the *Coprinus* peroxidase was determined based on the molar absorptivities of CIP at 405 nm (= 109 mM^{-1} cm^{-1}; Andersen *et al.*, 1991) as 3.85×10^{-11} mol U^{-1}. Additionally, the hydrogen peroxidase concentration at which maximum peroxidase activity occurs can be given by the following equation:

$$[H_2O_2]_{\max R} = \left(\frac{\beta}{\gamma} \right)^{1/2} = \left(\frac{b}{c} \right)^{1/2} \qquad \text{(A3-5)}$$

The kinetic parameters presented in Chapter 6 were estimated by regression using the data shown in Tables A3.1, A3.2, A3.3 and A3.4. For derivation of model equations, refer Nicell and Wright (1997).

Table A3.1. Apparent and relative peroxidase activity at various H_2O_2 concentrations (crude peroxidase from *Coprinus* sp. UAMH 10067).

	Apparent			Relative		
H_2O_2 (M)	0.014 U mL^{-1}	0.028 U mL^{-1}	0.051 U mL^{-1}	0.014 U mL^{-1}	0.028 U mL^{-1}	0.051 U mL^{-1}
0.00002	0.00303	0.00624	0.01028	0.213	0.226	0.202
0.00004	0.00605	0.01151	0.02417	0.425	0.418	0.474
0.00008	0.01007	0.01905	0.03760	0.707	0.691	0.738
0.00012	0.01316	0.02413	0.04564	0.924	0.875	0.895
0.00016	0.01362	0.02847	0.05005	0.956	1.033	0.982
0.0002	**0.01424**	**0.02756**	**0.05098**	**1.000**	**1.000**	**1.000**
0.0003	0.01278	0.02443	0.04824	0.897	0.886	0.946
0.0004	0.01056	0.01986	0.03784	0.741	0.721	0.742
0.0008	0.00648	0.01269	0.02226	0.455	0.460	0.437
0.0012	0.00497	0.00878	0.01731	0.349	0.319	0.339
0.0016	0.00363	0.00702	0.01057	0.255	0.255	0.207
0.002	0.00314	0.00578	0.00990	0.221	0.210	0.194

Table A3.2. Apparent and relative peroxidase activity at various H_2O_2 concentrations (purified peroxidase from *Coprinus* sp. UAMH 10067).

	Apparent			Relative		
H_2O_2 (M)	0.012 U mL^{-1}	0.017 U mL^{-1}	0.037 U mL^{-1}	0.014 U mL^{-1}	0.019 U mL^{-1}	0.038 U mL^{-1}
0.000016	0.00255	0.00350	0.00538	0.207	0.207	0.147
0.00002	0.00297	0.00389	0.00644	0.242	0.230	0.176
0.00004	0.00544	0.00714	0.01376	0.442	0.422	0.375
0.00008	0.00849	0.01201	0.02437	0.691	0.709	0.665
0.00012	0.01155	0.01445	0.03141	0.940	0.853	0.857
0.00016	0.01166	0.01567	0.03555	0.949	0.925	0.970
0.0002	**0.01229**	**0.01693**	**0.03665**	**1.000**	**1.000**	**1.000**
0.0003	0.01052	0.01433	0.03338	0.856	0.846	0.911
0.0004	0.00844	0.01211	0.02623	0.687	0.715	0.716
0.0008	0.00517	0.00679	0.01500	0.421	0.401	0.409
0.0012	0.00390	0.00543	0.01064	0.317	0.321	0.290
0.0016	0.00289	0.00380	0.00822	0.235	0.224	0.224
0.002	0.00251	0.00365	0.00657	0.204	0.216	0.179

Table A3.3. Apparent and relative peroxidase activity at various H_2O_2 concentrations (crude peroxidase from *C. cinereus* UAMH 4103).

	Apparent			Relative		
H_2O_2 (M)	0.016 U mL^{-1}	0.023 U mL^{-1}	0.037 U mL^{-1}	0.016 U mL^{-1}	0.023 U mL^{-1}	0.037 U mL^{-1}
0.000016	0.00289	0.00453	0.00676	0.184	0.197	0.181
0.00002	0.00351	0.00606	0.00727	0.223	0.263	0.194
0.00004	0.00665	0.01115	0.01494	0.422	0.484	0.400
0.00008	0.01117	0.01700	0.02625	0.709	0.738	0.702
0.00012	0.01354	0.02098	0.03321	0.860	0.911	0.888
0.00016	0.01495	0.02271	0.03436	0.949	0.987	0.919
0.0002	**0.01574**	**0.02302**	**0.03738**	**1.000**	**1.000**	**1.000**
0.0003	0.01359	0.02031	0.03448	0.863	0.882	0.923
0.0004	0.01170	0.01742	0.02837	0.743	0.757	0.759
0.0008	0.00635	0.00969	0.01570	0.404	0.421	0.420
0.0012	0.00463	0.00694	0.01176	0.294	0.302	0.315
0.0016	0.00358	0.00530	0.00839	0.227	0.230	0.224
0.002	0.00322	0.00510	0.00754	0.205	0.222	0.202

Table A3.4. Apparent and relative peroxidase activity at various H_2O_2 concentrations (purified peroxidase from *C. cinereus* UAMH 4103).

	Apparent			Relative		
H_2O_2 (M)	0.014 U mL^{-1}	0.019 U mL^{-1}	0.038 U mL^{-1}	0.014 U mL^{-1}	0.019 U mL^{-1}	0.038 U mL^{-1}
0.000016	0.00309	0.00451	0.00807	0.221	0.233	0.210
0.00002	0.00349	0.00590	0.00937	0.250	0.305	0.244
0.00004	0.00675	0.00844	0.01555	0.483	0.436	0.405
0.00008	0.01058	0.01391	0.02888	0.758	0.718	0.752
0.00012	0.01237	0.01697	0.03395	0.887	0.876	0.884
0.00016	0.01369	0.01818	0.03681	0.981	0.938	0.958
0.0002	**0.01396**	**0.01937**	**0.03840**	**1.000**	**1.000**	**1.000**
0.0003	0.01278	0.01725	0.03606	0.916	0.891	0.939
0.0004	0.01081	0.01426	0.02717	0.775	0.736	0.707
0.0008	0.00556	0.00783	0.01361	0.398	0.404	0.354
0.0012	0.00327	0.00544	0.00983	0.235	0.281	0.256
0.0016	0.00290	0.00426	0.00741	0.208	0.220	0.193
0.002	0.00254	0.00372	0.00674	0.182	0.192	0.175

References

Andersen, M. B., Hsuanyu, Y., Welinder, K. G., Schneider, P. and Dunford, H. B. (1991). Spectral and kinetic properties of oxidized intermediates of *Coprinus cinereus* peroxidase. *Acta Chemica Scandinavica* **45**: 1080-1086.

Nicell, J. A. (1994). Kinetics of horseradish peroxidase-catalyzed polymerization and precipitation of aqueous 4-chlorophenol. *Journal of Chemical Technology and Biotechnology* **60**: 203-215.

Nicell, J. A. and Wright, H. (1997). A model of peroxidase activity with inhibition by hydrogen peroxide. *Enzyme and Microbial Technology* **21**: 302-310.

A3.2. Response surface analyses data (Chapter 7, Figures 1 and 2)

Table A3.5. Complete data of the central composite design experiment for peroxidase production by *Coprinus* sp. UAMH 10067.

	Maximum peroxidase activity (U mL^{-1})				
ID#	#1	#2	#3	#4	Average
1	21.2	21.5	21.2		21.3
2	36.0	35.0	34.3		35.1
3	23.6	20.9	19.0		21.2
4	25.0	26.6	25.0		25.5
5	31.1	27.5	27.0		28.5
6	20.5	20.3	18.2		19.7
7	30.5	31.3	29.0		30.3
8	29.5	24.8	25.0		26.5
9	28.6	27.4	25.7	25.8	26.9
10	20.6	22.2	20.9		21.3
11	25.3	28.9	26.2		26.8
a	20.2	22.8	21.8		21.6
b	27.9	26.6	28.8		27.7
c	31.6	29.6	32.7		31.3
d	19.3	21.3	20.8		20.5

Table A3.6. Complete data of the central composite design experiment for peroxidase production by *C. cinereus* UAMH 4103.

ID#	Maximum peroxidase activity ($U\ mL^{-1}$) #1	#2	#3	Average
1	27.7	30.7	33.4	30.6
2	36.9	37.6	43.2	39.2
3	62.8	61.1	57.7	60.5
4	72.5	71.6	75.3	73.1
5	42.4	41.1	43.1	42.2
6	57.6	55.6	57.6	56.9
7	ND	ND	ND	ND
8	59.8	65.9	65.0	63.6
9	55.2	59.2	61.4	58.6
a	35.3	36.4	38.1	36.6
b	56.4	54.6	55.2	55.4
c	49.1	52.3	49.6	50.3
d	49.2	46.6	47.9	47.9

Note: ND = No data

Appendix 4. MALDI-TOF Mass Spectra

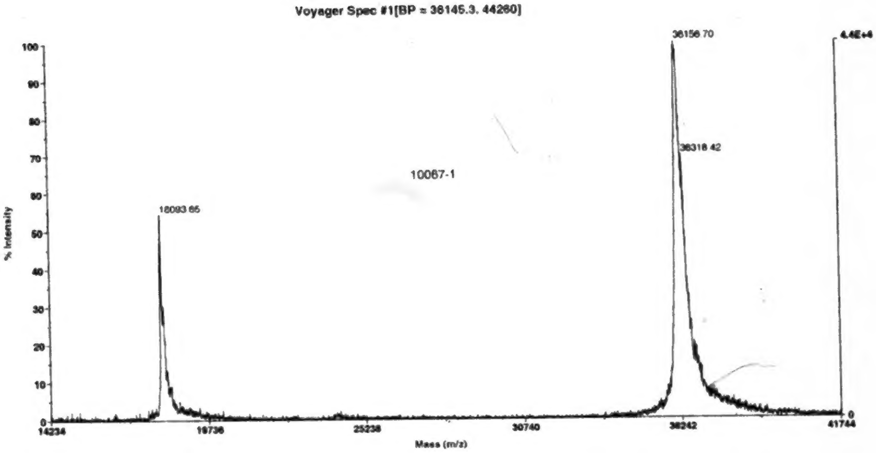


Figure A4.1. MALDI-TOF mass spectrum of peroxidase from *Coprinus* sp. UAMH 10067 (FPLC fraction #1).

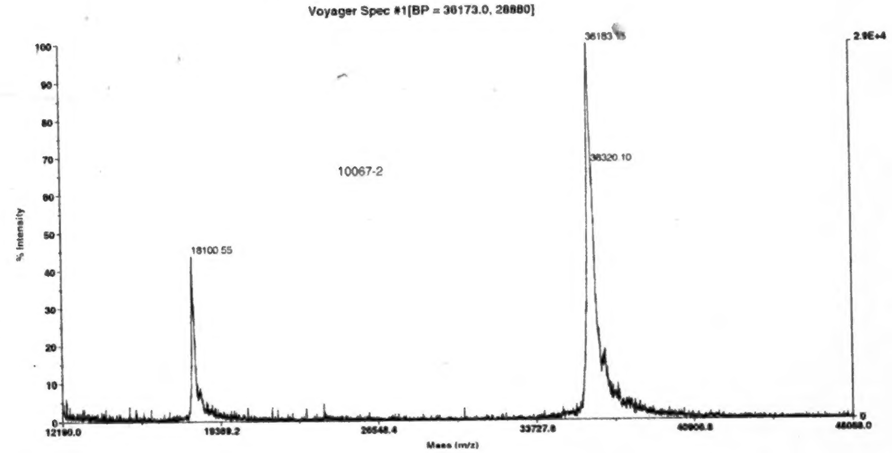


Figure A4.2. MALDI-TOF mass spectrum of peroxidase from *Coprinus* sp. UAMH 10067 (FPLC fraction #2).

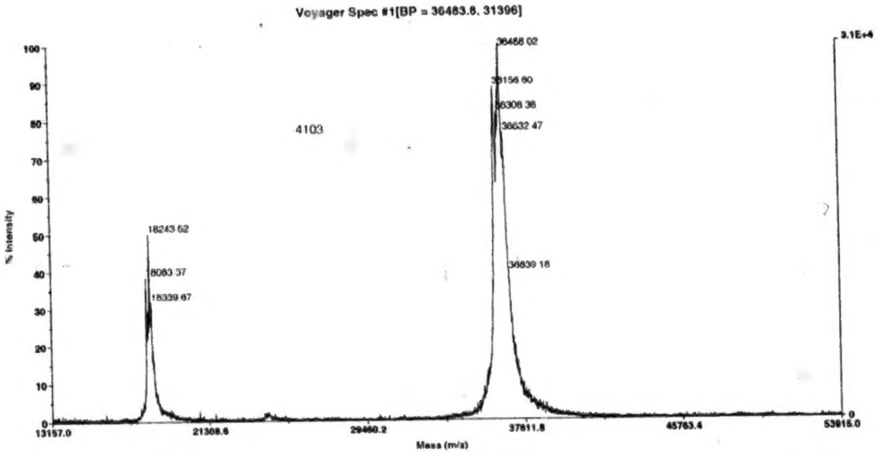


Figure A4.3. MALDI-TOF mass spectrum of peroxidase from *C. cinereus* UAMH 4103.

Appendix 5. Amino Acid Analyses Data

Table A5.1. Amino acid analyses data for peroxidases from *Coprinus* sp. UAMH 10067 and *C. cinereus* UAMH 4103.

	UAMH 10067 #1			UAMH 4103		
	mol	mol/mol	# Residue	mol	mol/mol	# Residue
Asp	3.93	0.101606	33.8	6.463	0.104949	34.9
Thr	2.107	0.054474	18.1	3.531	0.057338	19.1
Ser	3.974	0.102743	34.2	6.432	0.104446	34.8
Glu	3.643	0.094185	31.4	5.787	0.093972	31.3
Gly	4.327	0.111869	37.3	6.977	0.113296	37.7
Ala	3.841	0.099305	33.1	6.153	0.099916	33.3
Cys	0	0	8.0*	0	0	8.0*
Val	2.115	0.054681	18.2	3.559	0.057793	19.2
Met	0.61	0.015771	5.3	1.098	0.01783	5.9
Ile	1.684	0.043538	14.5	2.749	0.04464	14.9
Leu	3.31	0.085576	28.5	5.423	0.088061	29.3
Tyr	0.52	0.013444	4.5	0.631	0.010247	3.4
Phe	2.103	0.054371	18.1	3.41	0.055373	18.4
His	0.594	0.015357	5.1	0.927	0.015053	5.0
Lys	0.571	0.014763	4.9	0.867	0.014079	4.7
Trp	0	0	2.0*	0	0	2.0*
Arg	1.839	0.047545	15.8	2.911	0.04727	15.7
Pro	3.511	0.090773	30.2	4.664	0.075736	25.2
Total	38.679	1	343.0*	61.582	1	343.0*

Note: * Total number of residues and the numbers of cysteine and trptophan residues were assumed to be the same as the reported values (343, 8 and 2, respectively).